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anticipated by Simonet et al., Cell (1997) 89:309-319 (hereinafter "Simonet"), and claims 32-35 are rejected under 35 U.S.C. §102(a) as being anticipated by WO 97/23614 (hereinafter "the '614 publication"). Finally, claim 36 was withdrawn from further consideration because, according to the Examiner, claim 36 is drawn to a non-elected invention.

## RESPONSE

Specification

In the Office Action, a substitute specification excluding claims was requested pursuant to 37 C.F.R. §1.125(a). Accordingly, Applicants submit herewith a substitute specification under 37 C.F.R. §1.125(a) reflecting the amendments presented in the Preliminary Amendment filed June 23, 1999. The substitute specification, as set forth under §1.125(a), only contains subject matter from the original specification and is accompanied by (1) a statement that the substitute specification contains no new matter, and (2) a marked-up copy showing the amendments to be made via the substitute specification relative to the specification at the time the original specification was filed. The substitute specification corresponds to pages 1-86 and page 91 of the specification as originally-filed and is exclusive of claims, drawings and sequence listing of the originally-filed specification.

In compliance with the request in the Office Action, amendments to the abstract of the disclosure are set forth herein in the enclosed substitute specification.

Applicants note and thank the Examiner for acknowledging the claim made for foreign priority in the instant application under 35 U.S.C. §119(a)-(d) to JP 54977, filed on February 20, 1995, and to JP 207508, filed on July 21, 1995. A certified English translation of each application was filed in parent application U.S.S.N. 08/915,004.

Claim Rejection Under 35 U.S.C. §112, Second Paragraph

Claim 35 is rejected under 35 U.S.C. §112, second paragraph, as being indefinite. Applicants' amendment to claims 35 should satisfy the Examiner's concern in this regard.

Claim Rejection under 35 U.S.C. §102

Claims 32-33 are rejected under 35 U.S.C. §102(a) as being anticipated by Simonet and claims 32-35 are rejected under 35 U.S.C. §102(a) as being anticipated by the '614 publication.

Applicants respectfully submit that the instant application claims priority to JP 54977/1995, filed on February 20, 1995 and JP 207508/1995, filed on July 21, 1995. These priority documents ante-date the publication date of Simonet (April 8, 1997) and the earliest priority date of the '614 publication (December 22, 1995). Consequently, neither Simonet nor the '614 publication are prior art to the instant application under 35 U.S.C. §102. Similar arguments were made in the parent application U.S.S.N. 08/915,004 to overcome older art cited in the parent application. Applicants respectfully request that the rejection be withdrawn.

**CONCLUSION**

Applicants respectfully urge, in view of the foregoing amendments and remarks, that all claims are in condition for allowance. Accordingly, Applicants respectfully request reconsideration of the elected claims, as amended, and prompt and favorable action in the application. If the Examiner believes a telephone conference with the undersigned representative would be helpful in expediting prosecution of this application, he is urged to call the undersigned at (617) 248-7044.

Respectfully submitted,

Ronda P. Moore, D.V.M.

Date: May 9, 2000
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SUBSTITUTE SPECIFICATION

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SPECIFICATION

# NOVEL PROTEINS AND METHODS FOR PRODUCING THE PROTEINS

## Field of the invention

This invention relates to a novel protein, osteoclastogenesis inhibitory factor (OCIF), and methods for producing the protein.

## Background of the invention

Human bones are always remodelling by the repeated process of resorption and reconstitution. [In the process, osteoblasts] Osteoblasts and osteoclasts are considered to be the cells mainly responsible for bone formation and bone resorption, respectively. A typical example of a disease caused by [the progression of] abnormal bone metabolism is osteoporosis. [The disease] Osteoporosis is known to [be provoked] result [by the condition in which] when bone resorption by osteoclasts exceeds bone formation by osteoblasts, but the mechanism of osteoporosis has not yet been completely elucidated. Osteoporosis causes bone pain [in the bone] and makes [the bone] bones fragile, leading to fracture, particularly in elderly patients. [fragile, leading to fracture. Since osteoporosis increases the number of bedridden old people, it has] Osteoporosis has therefore become a social issue with the increasing number of [old] elderly people in the population. Therefore, [efficacious] effective drugs for the treatment of the disease are expected to be developed. Bone mass reduction caused by [the] abnormal bone metabolism is thought to be prevented by inhibiting bone resorption, improving bone formation, or improving the [balanced] balance of bone metabolism.

Bone formation is [expected to be] promoted by stimulating growth, differentiation, or activation of osteoblasts. Many cytokines [are reported] reportedly [to] stimulate growth or [differentation] differentiation of osteoblasts, i.e. fibroblast growth

factor (FGF) (Rodan S.B. et al., Endocrinology vol.121, p1917, 1987), insulin-like growth factor-I (IGF-I) (Hock J.M. et al., Endocrinology vol. 122, p254, 1988), insulin-like growth factor-II (IGF-II) (McCarthy T. et al., Endocrinology vol.124, p301, 1989), Activin A (Centrella M. et al., ~~Mol, Cell,~~ Mol. Cell. Biol. vol. 11, p250, 1991), Vasculotropin (Varonique M. et al., Biochem. Biophys. Res. Commun. vol. 199, p380, 1994), and bone morphogenetic protein (BMP) (Yamaguchi, A. et al., J. Cell Biol. vol. 113, p682, 1991, Sampath T.K. et al., J. Biol. Chem. vol.267, p20532, 1992, and Knutsen R. et al., Biochem. Biophys. Res. Commun. vol.194, p1352, ~~1993.~~ 1993).

On the other hand, cytokines which ~~inhibits~~ inhibit differentiation and/or maturation of osteoclasts ~~have been paid attention and have~~ have also been intensively studied. Transforming growth factor-$\beta$ (Chenu C. et al., Proc. Natl. Acad. Sci. USA, vol.85, p5683, 1988) and interleukin-4 (Kasano K. et al., Bone-Miner., vol. 21, p179, 1993) ~~are found to~~ inhibit the differentiation of osteoclasts. Calcitonin (Bone-Miner., vol.17, p347, 1992), ~~Macrophage~~ macrophage colony-stimulating factor (Hattersley G. et al. J. Cell. Physiol. vol.137, p199, 1988), interleukin-4 (Watanabe, K. et al., Biochem. Biophys. Res. Commun. vol. 172, p1035, 1990), and interferon-$\gamma$ (Gowen M. et al., J. Bone Miner. Res., vol.1, p469, 1986) ~~are found to~~ inhibit bone resorption by osteoclasts.

These cytokines are expected to be ~~efficacious~~ effective drugs for improving bone mass reduction by stimulating bone formation and/or by inhibiting bone resorption. ~~The cytokines~~ Cytokines such as ~~insulin like~~ insulin-like growth factor-I and bone morphogenetic proteins ~~are now~~ have been investigated in clinical trials for their

effectiveness for treating effects in treatment of patients with bone diseases. Calcitonin is already used as a drug to treat to care osteoporosis and to diminish pain in osteoporosis. patients

Examples of drugs now clinically utilized for the treatment of bone diseases and for shortening the treatment period are dihydroxyvitamine $D_3$, vitamin $K_2$, calcitonin and its derivatives, hormones such as estradiol, ipriflavon, and calcium preparations. preparations . However, these drugs do not provide satisfactory therapeutic effects, and novel drug substances are have been expected to be developed. Since As mentioned, bone metabolism is manifest controlled in the balance between bone resorption and bone formation, formation. Therefore, cytokines which inhibit osteoclast differentiation and/or maturation are expected to be developed as drugs for the treatment of bone diseases such as osteoporosis.

## Summary Disclosure of Invention

This invention was initiated from the view point described above. The The purpose of this invention is to offer both a novel factor, termed osteoclastogenesis inhibitory factor (OCIF), and a procedure to produce the factor efficiently.

The inventors have intensively searched for osteoclastogenesis inhibitory factors in human embryonic fibloblast IMR-90 (ATCC CCL186) conditioned medium and have found a novel osteoclastogenesis inhibitory factor (OCIF) which inhibits differentiation and/or maturation of osteoclasts.

The inventors have established a method for accumulating the protein to a high concentration by culturing IMR-90 cells on using alumina ceramic pieces, which function as the cell adherence matrices.

The inventors have also established an efficient method for isolating the protein, OCIF, from the IMR-90 conditioned medium using the following sequential column Chromatography: chromatography, ion-exchange, heparin affinity, cibacron-blue affinity, and reverse phase.

After determining The inventors, based on the amino acid sequence of the purified natural OCIF, a cDNA encoding this protein was successfully cloned. OCIF, successfully cloned a cDNA encoding this protein. The inventors A procedure for producing this protein was also established. established also a procedure to produce this protein which inhibits differentiation of osteoclasts. The This invention concerns a protein which is produced by human lung fibroblast cells, is has molecular weight by weights in SDS-PAGE of 60kD 60 KD under In the reducing conditions and 120kD 120 KD under the non-reducing conditions, and has affinity for both cation-exchange resins and heparin. heparin, reduces its activity The proteins ability to inhibit the differentiation and maturation of osteoclasts is reduced when if treated for 10 minutes at 70 ℃ or for 30 minutes at 56 ℃, and lose its activity its ability to inhibit differentiation and maturation of osteoclasts is lost when treated by the treatment for 10 minutes at 90 ℃. The amino acid sequence of the OCIF protein of protein OCIF which is described in the present invention is clearly different from any other factors known to inhibit the of know factors inhibiting formation of osteoclasts.

The invention includes a method for purifying to purify OCIF protein, comprising: comprising ; (1) culturing human fibroblasts, (2) applying the conditioned medium to a heparin column to obtain the adsorbed fraction, (3) purifying the OCIF protein using a cation-exchange column, (4) purifying the OCIF protein using a heparin affinity column, (5) purifying the OCIF protein using a cibacron blue affinity column, and (6) isolating the OCIF protein using reverse-phase column chromatography. Cibacron blue F3GA may be coupled to a carrier made of synthetic

hydrophilic polymers, for example.

[hydrophilic polymers is an example of materials used to prepare Cibacron blue] [columns.] These columns are conventionally called "blue [colomns]". columns

The invention includes a method for [accumulating the] producing OCIF protein [to a] in high concentration by culturing human fibroblasts using alumina ceramic pieces as the cell-adherence matrices.

Moreover, the inventors determined the amino acid sequences of [the] peptides derived from OCIF, designed the oligonucleotide primers based on these amino acid sequences, and obtained cDNA fragments encoding OCIF from a cDNA library of IMR-90 cells. The full length OCIF cDNA encoding the OCIF protein is cloned from a cDNA library using an OCIF DNA fragment as a probe. The OCIF cDNA containing the entire coding region is inserted into an expression vector. Recombinant OCIF can be produced by expressing the OCIF cDNA, containing the entire coding region, in mammalian cells or bacteria. *See Sheet 5A attached

Detailed description of the invention

The OCIF protein of the present invention can be isolated from human fibroblast conditioned medium with high yield. The procedure to isolate OCIF is based on ordinary techniques for purifying proteins from biomaterials, in accordance with the physical and chemical properties of OCIF protein. For example, concentrating procedures include [procedure includes] ordinary biochemical techniques such as ultrafiltration, lyophilization [lyophylization], and dialysis. Purifying procedures include [procedure includes] combinations of several chromatographic techniques for purifying proteins such as ion-exchange column chromatography, affinity column chromatography, gel filtration column chromatography, hydrophobic column chromatography, reverse phase column chromatography, and preparative gel electrophoresis. The human fibroblasts used for the production of OCIF protein are preferably IMR-90 cells. A [fibroblast used for production of the OCIF protein is preferably IMR-90. A] method for producing [the] IMR-90 conditioned medium is preferably a process comprising, adhering human embryonic fibroblast IMR-90 cells to alumina

Text to be inserted before "Detailed description of the invention:

## Brief Description of the Figures

Figure 1 shows the elution pattern of crude OCIF protein (HiLoad-Q/FF pass-through fraction; sample 3) from a HiLoad-S/HP column.

Figure 2 shows the elution pattern of crude OCIF protein (heparin 5PW fraction; sample 5) from a blue-5PW column.

Figure 3 shows the elution pattern of OCIF protein (blue-5PW fraction 49 to 50) from a reverse-phase column.

Figures 4A and 4B show the SDS-PAGE of isolated OCIF proteins under reducing or non-reducing conditions. Description of the lanes:

lane 1, 4: molecular weight marker proteins;
lane 2, 5: OCIF protein of peak 6 in Figure 3;
lane 3, 6: OCIF protein of peak 7 in Figure 3.

Figure 5 shows the elution pattern of peptides obtained by the digestion of pyridyl ethylated OCIF protein digested with lysylendopeptidase on a reverse-phase column.

Figure 6 shows the SDS-PAGE of isolated natural (n) OCIF protein and recombinant (r) OCIF proteins under non-reducing conditions. rOCIF(E) and rOCIF(C) proteins were produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 1: molecular weight marker proteins;
lane 2: a monomer type nOCIF protein;
lane 3: a dimer type nOCIF protein;
lane 4: a monomer type rOCIF(E) protein;
lane 5: a dimer type rOCIF(E) protein;
lane 6: a monomertype rOCIF(C) protein;
lane 7: a dimer type rOCIF(C) protein.

Figure 7 shows the SDS-PAGE of isolated natural (n) OCIF proteins and recombinant (r) OCIF proteins under reducing conditions. rOCIF (E) and rOCIF (C) were produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 8: molecular weight marker proteins;
lane 9: a monomer type nOCIF protein;
lane 10: a dimer type nOCIF protein;
lane 11: a monomer type rOCIF(E) protein;
lane 12: a dimer type rOCIF(E) protein;
lane 13: a monomer type rOCIF (C) protein;
lane 14: a dimer type rOCIF(C) protein.

Figure 8 shows the SDS-PAGE of isolated natural (n) OCIF proteins and recombinant (r) OCIF proteins from which N-linked sugar chains were removed under reducing conditions. rOCIF (E) and rOCIF(C) are rOCIF proteins produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 15: molecular weight marker proteins;
lane 16: a monomer type nOCIF protein;
lane 17: a dimer type nOCIF protein;
lane 18: a monomer type rOCIF(E) protein;
lane 19: a dimer type rOCIF(E) protein;
lane 20: a monomer type rOCIF(C) protein;
lane 21: a dimer type rOCIF (C) protein.

Figure 9 shows a comparison of OCIF and OCIF2 amino acid sequences.

Figure 10 shows a comparison of OCIF and OCIF3 amino acid sequences.

Figure 11 shows a comparison of OCIF and OCIF4 amino acid sequences.

Figure 12 shows a comparison of OCIF and OCIF5 amino acid sequences.

Figure 13 shows a standard curve determining OCIF protein concentration by an EIA employing anti-OCIF polyclonal antibodies.

Figure 14 shows a standard curve determining OCIF protein concentration by an EIA employing anti-OCIF monoclonal antibodies.

Figure 15 shows the effect of r-OCIF protein on model rats with osteoporosis.

in

ceramic pieces in roller-bottles[, using] DMEM medium supplemented with 5 % new born calf serum [for the cell culture] and cultivating the cells in roller-bottles for 7 to 10 days by stand cultivation. CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) is preferably [prefarably] added to the buffer as a detergent in the [purification steps of OCIF protein.] protein purification procedure.

The OCIF protein of the instant invention can be obtained initially [initially obtained] as a basic heparin binding [heparin binding basic] OCIF fraction by applying the culture medium to a heparin column (Heparin-Sepharose CL-6B, Pharmacia), eluting with 10 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl, and [then by] applying the OCIF fraction to a Q・anion-exchange column (HiLoad-Q/FF, Pharmacia), and collecting the non-adsorbed fraction. OCIF protein can be purified by subjecting the obtained OCIF fraction to purification on a S・cation-exchange column (HiLoad-S/FF, Pharmacia)[.], a heparin column (Heparin-5PW, TOSOH), a Cibacrone Blue column (Blue-5PW, TOSOH), and a reverse-phase column (BU-300 C4, Perkin [Elmer) and] Elmer). [the material is defined by the previously described properties.]

The present invention relates to a method of cloning cDNA encoding the OCIF protein based on the amino acid sequence of natural OCIF and a method for [method of] obtaining recombinant OCIF [protein that inhibits differentiation and/or] protein. [maturation of osteoclasts.] The OCIF protein is purified according to the method described in the present invention and is treated with endopeptidase (for example, lysylendopeptidase). The amino acid sequences of the peptides produced by the digestion are determined and the mixture of oligonucleotides

is synthesized.

that can encode each internal amino acid sequence [was systhesized.] The OCIF cDNA fragment is obtained by PCR (preferably RT-PCR, reverse transcriptase PCR) using the oligonucleotide mixtures described above as primers. The full length OCIF cDNA encoding the OCIF protein is cloned from a cDNA library using [the] an [obtained] OCIF DNA fragment as a probe. The OCIF cDNA containing the entire coding region is inserted into an expression vector. Recombinant [The recombinant] OCIF can be produced by expressing the OCIF cDNA, containing the entire coding region, in mammalian cells or bacteria.

The present invention relates to the novel proteins OCIF2, OCIF3, OCIF4, and OCIF5 that are variants of OCIF and have the activity described above. These OCIF variants are obtained from the cDNA library constructed with IMR-90 poly(A) + RNA [by hybridization] using the OCIF cDNA fragment as a hybridization probe. Each of the OCIF variant cDNAs containing the entire coding region is inserted into an expression vector. Each recombinant OCIF variant protein can be produced by expressing each of the OCIF variant cDNAs, containing the entire coding region, in [the] conventional hosts. Each recombinant OCIF variant protein can be purified according to the method described in this invention. Each recombinant OCIF variant protein has the [an] ability to inhibit osteoclastogenesis.

The present invention further includes OCIF mutants. They are substitution mutants comprising the replacement of one cysteine residue, possibly involved in dimer formation, with a serine residue or [formation with serine residue, and] various deletion mutants of OCIF. Substitutions or deletions are introduced into the OCIF cDNA using

polymerase chain reaction (PCR) or [by] restriction enzyme digestion. Each of these mutated OCIF cDNAs is inserted into a vector [containing] having an appropriate promoter for gene expression. The resultant expression vector for each of the OCIF mutants is transfected into eukaryotic cells such as mammalian cells. Each of OCIF mutants can be obtained and purified from the conditioned media of the transfected cells.

The present invention provides polyclonal antibodies and a method to quantitatively determine OCIF concentration using these polyclonal antibodies. [As antigens (immunogens), natural] Natural OCIF obtained from IMR-90 conditioned medium, recombinant OCIF produced by such hosts as microorganisms and eukaryotes using OCIF cDNA, synthetic peptides [designed] based on the amino acid sequence of OCIF, or peptides obtained from OCIF by partial digestion can be [used.] used as antigens. Anti-OCIF polyclonal antibodies are obtained by immunizing appropriate mammals with the antigens, in combination with adjuvants [for immunization] if necessary, [purifying from the serum by the] and purifying the antibodies from the serum by ordinary purification methods. [The anti-OCIF] Anti-OCIF polyclonal antibodies which are labelled with [rasioisotopes] radioisotopes or enzymes can be used in radio-immunoassay (RIA) [system or immunoassay (EIA) system.] systems or enzyme-immunoassay (EIA) systems. [By using] Using these assay systems, the [concentrations] concentration of OCIF in biological materials such as [blood and] blood, ascites and [cells-culture] cell-culture medium can be easily determined.

[The antibodies in the present invention can be used in radio immunoassay (RIA) or enzyme immunoassay (EIA). By using these assay systems, the concentration of OCIF in biological materials such as blood and ascites can]

be easily determined.

The present invention provides novel monoclonal antibodies and a method
for determining
to quantitatively determine OCIF concentration using these monoclonal antibodies.

Anti-OCIF monoclonal antibodies can be produced by the conventional
methods
method using OCIF as an antigen. Native OCIF obtained from the culture medium of IMR-90 cells and recombinant OCIF produced by such hosts as microorganisms
transfected with
and eukaryotes using OCIF cDNA can be used as antigens. Alternatively,
synthetic peptides
synthesized peptides designed based on the amino acid sequence of OCIF and peptides obtained from OCIF by partial digestion can be also used as antigens.
immunizing mammals such as mice or rats
Immunized lymphocytes obtained by immunization of mammals with the antigen or
mammalian myeloma cells
by an in vitro immunization method were fused with myeloma of mammals to
hybridomas.
antibodies
recognize
obtain hybridoma. The hybridoma clones secreting antibody which recognizes
OCIF were selected and cultured to obtain the desired antibodies.
OCIF were selected from the hybridomas obtained by the cell fusion. The desired antibodies can be obtained by cell culture of the selected hybridoma clones. In preparation of hybridoma, small animals such as mice or rats are
For immunizations, OCIF is suitably diluted with
generally used for immunization. To immunize, OCIF is suitably diluted with a saline solution (0.15 M NaCl), and is intravenously or intraperitoneally administered with an adjuvant to animals for 2 -5 times every 2 -20 days. The
the
immunized animal was killed three days after final immunization, the spleen
removed
was taken out and the splenocytes were used as immunized B lymphocytes.

useful
Mouse myeloma cell lines for cell fusion with the immunized B lymphocytes include, for example, p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, FO, p3x63

Ag8.653, and S194 cells. The rat cell line R-210 may also be used. Alternatively, human B lymphocytes

Ag8.653, and S194. Rat R-210 cell line may also be used. Human B lymphocytes are immunized by an in vitro immunization method and are fused with human

cells

myeloma cell line or EB virus transformed human B lymphocytes which are used

to produce human type antibodies.

as a parent cell line for cell fusion, to produce human type antibody.

cells

Cell fusion of the immunized B lymphocytes and myeloma cell line is carried out principally by the conventional methods. For example, the method

used. Alternatively,

of Koehler G. et al. (Nature 256, 495-497, 1975) is generally used, and also

used.

an electric pulse method can be applied to cell fusion. The immunized B lymphocytes and transformed B cells are mixed at conventional ratios and a cell culture medium without FBS containing polyethylene glycol is generally

used to fuse the cells. The fusions products are

used for cell fusion. The B lymphocytes fused with myeloma cell lines are cultured in HAT selection medium containing FBS to select hybridoma. hybridomas.

An EIA, plaque

For screening of hybridoma producing anti-OCIF antibody, EIA, plaque

used to screen for hybridomas producing anti-OCIF antibodies

assay, Ouchterlony, or agglutination assay can be principally used. Among

EIA is a simple assay which is easy to perform

them, EIA is simple and easy to operate with sufficient accuracy and is

therefore generally used. The desired antibody can be

generally used. By EIA using purified OCIF, the desired antibody can be

using EIA and purified OCIF. Hybridoma obtained the

selected easily and accurately. Thus obtained hybridoma can be cultured by the

methods

conventional method of cell culture and frozen for stock if necessary. The

cells using

antibody can be produced by culturing hybridoma using the ordinary cell

methods cells into live

culture method or by transplanting hybridoma intraperitoneally to animals. The antibody can be purified by the ordinary purification methods such as salt precipitation, gel filtration, and affinity chromatography. The obtained

obtained

antibody specifically reacts with OCIF and can be used for determination of to determin

to purify OCIF protein.

OCIF concentration and for purification of OCIF. The antibodies of the

present invention recognize epitopes of OCIF and have high affinity [to] for OCIF. Therefore, they can be used for the construction of EIA. [By (using) this assay system,] this assay system is useful for determining the concentration of OCIF in biological materials such as [blood and ascites can be easily determined.] blood and ascites.

[The agents used for treating bone diseases that contain OCIF as an] The present invention provides agents, containing OCIF as an effective [ingredient are provided by the present invention.] ingredient, that are useful for treating bone diseases. Rats were subjected to denervation of the left forelimb. Test compounds were administered daily after surgery for 14 days. After 2 weeks of treatment, the animals were sacrificed and their forelimbs were dissected. Thereafter bones were tested for mechanical strength by the three point bending method. OCIF improved the mechanical strength of bone in a dose dependent manner.

The OCIF protein of the invention is useful as a pharmaceutical [ingredients] ingredient for treating or improving decreased bone mass in bone diseases such as osteoporosis, [bone diseases such as] rheumatism, osteoarthritis, and abnormal bone metabolism in multiple myeloma. [The] OCIF protein is also useful as an antigen [to establish] in the immunological diagnosis of [the] bone diseases. Pharmaceutical preparations containing [the] OCIF protein as an active [ingredients] ingredient are formulated and can be orally or parenterally administered. The preparation contains the OCIF protein of the present invention as an [efficacious] effective ingredient and is safely administered to [human] humans and animals. Examples of [the] pharmaceutical preparations include compositions for injection or intravenous drip, suppositories, nasal preparations, sublingual preparations, and tapes for percutaneous absorption. The pharmaceutical preparation for injection can

a effective

be prepared by mixing the pharmacologically efficacious amount of OCIF protein

a carrier.

and pharmaceutically acceptable carriers. The carriers are vehicles and/or activators, e.g. amino acids, saccharides, cellulose derivatives, and other organic and inorganic compounds, which are generally added to active ingredients. When the OCIF protein is mixed with the vehicles and/or

activators for injection, pH adjusters, buffers, stabilizers,

activators to prepare injections, pH adjuster, buffer, stabilizer,

by conventional methods

solubilizing agent, etc. can be added, if necessary.

Brief description of the figures

Figure 1 shows the elution pattern of crude OCIF protein (Hiload-Q/FF pass-through fraction ; sample 3) from a Hiload-S/HP column.

Figure 2 shows the elution pattern of crude OCIF protein (heparin-5PW fraction ; sample 5) from a blue-5PW column.

Figure 3 shows the elution pattern of OCIF protein (blue-5PW fraction 49 to 50) from a reverse-phase column.

Figure 4 shows the SDS-PAGE of isolated OCIF proteins under reducing conditions or non-reducing conditions.

Description of the lanes,

lane 1,4 ; molecular weight marker proteins

lane 2,5 ; OCIF protein of peak 6 in figure 3

lane 3,6 ; OCIF protein of peak 7 in figure 3

Figure 5 shows the elution pattern of peptides obtained by the digestion of pyridyl ethylated OCIF protein digested with lysylendopeptidase, on a reverse-phase column.

Figure 6 shows the SDS-PAGE of isolated natural(n) OCIF protein and recombinant(r) OCIF proteins under non-reducing conditions. rOCIF(E) and rOCIF(C) were produced in 293/EBNA cells and in CHO cells, respectively.

Description of the lanes,

lane 1 ; molecular weight marker proteins

lane 2 ; a monomer type nOCIF protein

lane 3 ; a dimer type nOCIF protein

lane 4 ; a monomer type rOCIF(E) protein

lane 5 ; a dimer type rOCIF(E) protein

lane 6 ; a monomer type rOCIF(C) protein

lane 7 ; a dimer type rOCIF(C) protein

Figure 7 shows the SDS-PAGE of isolated natural(n) OCIF proteins and recombinant (r) OCIF proteins under reducing conditions. rOCIF(E) and rOCIF(C) were produced in 293/EBNA cells and in CHO cells, respectively.

Description of the lanes,

lane 8 ; molecular weight marker proteins

lane 9 ; a monomer type nOCIF protein

lane 10 ; a dimer type nOCIF protein

lane 11 ; a monomer type rOCIF(E) protein

lane 12 ; a dimer type rOCIF(E) protein

lane 13 ; a monomer type rOCIF(C) protein

lane 14 ; a dimer type rOCIF(C) protein

Figure 8 shows the SDS-PAGE of isolated natural(n) OCIF proteins and recombinant(r) OCIF proteins from which N-linked sugar chains were removed

under reducing conditions. rOCIF(E) and rOCIF(C) are rOCIF protein produced in 293/EBNA cells and in CHO cells, respectively.

Description of the lanes,

lane 15 ; molecular weight marker proteins

lane 16 ; a monomer type nOCIF protein

lane 17 ; a dimer type nOCIF protein

lane 18 ; a monomer type rOCIF(E) protein

lane 19 ; a dimer type rOCIF(E) protein

lane 20 ; a monomer type rOCIF(C) protein

lane 21 ; a dimer type rOCIF(C) protein

Figure 9 shows comparison of amino acid sequences between OCIF and OCIF2.

Figure 10 shows comparison of amino acid sequences between OCIF and OCIF3.

Figure 11 shows comparison of amino acid sequences between OCIF and OCIF4.

Figure 12 shows comparison of amino acid sequences between OCIF and OCIF5.

Figure 13 shows standard curve for determination of OCIF protein concentration by an EIA employing anti-OCIF polyclonal antibodies.

Figure 14 shows standard curve for determination of OCIF protein concentration by an EIA employing anti-OCIF monoclonal antibodies.

Figure 15 shows the effect of rOCIF protein on osteoporosis.

Best Mode for Carrying Out the Invention

The present invention will be further explained by the following examples, however, though the scope of the invention is not restricted to the examples. thereto.

## EXAMPLE 1

Preparation of a conditioned medium of human fibroblast IMR-90

Human fetal lung fibroblast IMR-90 (ATCC-CCL186) cells were cultured on alumina ceramic pieces (80 g) (alumina: 99.5%, manufactured by Toshiba Ceramic K.K.) in DMEM medium (manufactured by Gibco BRL Co.) supplemented with 5% CS and 10mM HEPES buffer (500 ml/roller bottle) at 37℃ in [under] the presence of 5% $CO_2$ for 7 to 10 days using 60 roller bottles (490 $cm^2$, 110 x 171mm, manufactured by Corning [Coning] Co.) in static culture. The conditioned medium was harvested[,] and a fresh medium was added to the roller bottles. About 30L of IMR-90 conditioned medium per batch culture was obtained. The conditioned medium was designated as sample 1.

## EXAMPLE 2

Assay method for osteoclast development inhibitory activity

Osteoclast development inhibitory activity was assayed by measuring tartrate-resistant acid phosphatase(TRAP) activity according to the methods of M. Kumegawa et.al (Protein・Nucleic Acid・Enzyme, vol.34 p999, 1989) and N. Takahashi et al. [et.al] (Endocrynology, vol.122, p1373, 1988 ) with modifications. Briefly, bone marrow cells obtained from a 17 day-old mouse were suspended in α-MEM (manufactured by GIBCO BRL Co.) containing 10% FBS, 1x10$^{-8}$M [2x10$^{-8}$M] of activated vitamin D3 and a test sample [$D_3$, and each test sample,] and were inoculated into [to] each well of a 96-well plate at a cell density of 3x10$^5$ cells/0.2 ml/well. The plates were incubated for 7 days at 37℃ in humidified 5%$CO_2$. Cultures were maintained [further continued] by replacing 0.16 ml of old medium with the same volume of fresh

medium on day 3 and day 5 after Cultivation began. starting cultivation. On day 7, the plates were washed after washing the plates with phosphate buffered saline, and the cells were fixed with ethanol/acetone (1:1) for 1 min. at room temperature. Osteoclast temperature, and then osteoclast development was tested by determining acid for phosphatase activity using a kit (Acid Phosphatase, Leucocyte, Catalog No. 387-A, manufactured by Sigma Co.). A decrease in the number The decrease of TRAP positive cells was taken as an indication of OCIF activity.

EXAMPLE 3

Purification of OCIF

i) Heparin Sepharose CL-6B column chromatography

The 90L of IMR-90 conditioned medium (sample 1) was filtered using a filtrated with 0.22 $\mu$ membrane filter (hydrophilic Milidisk, 2000 $cm^2$, Milipore Co.), and was divided into three 30 liter portions. Each portion (30 l) was applied to a heparin Sepharose CL-6B column (5 x 4.1 cm, Pharmacia Co.) equilibrated with 10mM Tris-HCl containing 0.3M NaCl, pH 7.5. After washing the column with 10mM Tris-HCl, pH 7.5 at a flow rate of 500 ml/hr., the heparin Sepharose CL-6B adsorbent protein fraction was eluted with 10mM Tris-HCl, pH 7.5, containing 2M NaCl. The fraction was designated as sample 2.

ii) HiLoad-Q/FF column chromatography

The heparin Sepharose-adsorbent fraction (sample 2) was dialyzed against 10mM Tris-HCl, pH 7.5, supplemented with CHAPS to a final concentration of 0.1%, incubated at 4 ℃ overnight, and divided into two portions. Each

portion was then applied to an anion-exchange column (HiLoad-Q/FF, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5 to obtain a non-adsorbent fraction (1000 ml). The fraction was designated [as] sample 3.

iii) HiLoad-S/HP column chromatography

The HiLoad-Q non-adsorbent fraction (sample 3) was applied to a cation-exchange column (HiLoad-S/HP, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 50 mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient from 0 to 1 M NaCl at a flow rate of 8 ml/min for 100 min. and fractions (12 ml) were collected. Every [Each] ten fractions from [number] numbers 1 to 40 were pooled [was pooled] to form one portion. [Each] 100 $\mu$l each of the four portions was tested for OCIF activity. OCIF activity was observed in fractions [from] 11 to 30 (as shown in Figure 1). Fractions 21 to 30, [The fractions from 21 to 30 which had higher specific activity were collected and was designated as sample 4.] which had higher specific activity, were pooled and designated sample 4.

iv) Heparin-5PW affinity column chromatography

One hundred and twenty ml of HiLoad-S fractions [fraction from] 21 to 30 (sample 4) was diluted with 240 ml of 50 mM Tris-HCl, 0.1% CHAPS, pH 7.5, and applied to a heparin-5PW affinity column (0.8 x 7.5 cm, Tosoh Co.) which was equilibrated with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient from 0 to 2M NaCl at a flow rate of 0.5ml/min for 60 min. and fractions

(0.5 ml) were collected. Fifty $\mu$l ~~was~~ were removed from each fraction to test for OCIF activity. The active fractions, eluted with 0.7 to 1.3M NaCl ~~was~~ were pooled and ~~was designated as~~ designated sample 5.

v) Blue 5PW affinity column chromatography

Ten ml of sample 5 ~~was~~ were diluted with 190 ml of 50mM Tris-HCl, 0.1% CHAPS, pH 7.5 and applied to a blue-5PW affinity column, (0.5x5 cm, Tosoh Co.) which was equilibrated with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 50mM Tris-HCl, 0.1% CHAPS, pH7.5, the adsorbed protein was eluted with a 30 ml linear gradient from 0 to 2M NaCl at a flow rate of 0.5 ml/min., and fractions (0.5 ml) were collected. Using 25 $\mu$l of each fraction, OCIF activity was evaluated. ~~The fractions number~~ Fractions 49 to 70, eluted with 1.0-1.6M NaCl, had OCIF activity.

vi) Reverse phase column chromatography

The blue 5PW fraction obtained by collecting fractions ~~from 49 to~~ 49 and 50 was acidified with 10$\mu$l of 25% TFA and applied to a reverse phase C4 column (BU-300, 2.1x220mm, manufactured by Perkin-Elmer) which was equilibrated with 0.1% of TFA and 25% ~~of~~ acetonitrile. The adsorbed protein was eluted with a linear gradient from 25 to 55% acetonitrile at a flow rate of 0.2 ml/min. for 60 min., and each protein peak was collected (Fig.3). One hundred $\mu$l of each peak fraction was tested for OCIF activity, and ~~peak 6 and the peak~~ peaks 6 and 7 had OCIF activity. The result was shown in Table 1.

Table 1

OCIF activity eluted from reverse phase C4 column

| Sample | Dilution | | | |
|---|---|---|---|---|
| | 1/40 | 1/120 | 1/360 | 1/1080 |
| Peak 6 | ++ | ++ | + | – |
| Peak 7 | ++ | + | – | – |

[ ++ means OCIF activity inhibiting osteoclast development more than 80%, + means OCIF activity inhibiting osteoclast development between 30% and 80%, and – means no OCIF activity.]

EXAMPLE 4

Molecular weight of OCIF protein

with OCIF activity (peaks 6 and 7)

The two protein peaks (6 and 7) with OCIF activity were subjected to SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Briefly, 20 $\mu$l of each peak fraction was concentrated under vacuum and dissolved in 1.5 $\mu$l of 10mM Tris-HCl, pH 8, 1mM EDTA, 2.5% SDS, 0.01% bromophenol blue, and incubated at 37℃ overnight under non-reducing conditions or under reducing conditions (with 5% of 2-mercaptoethanol). Each 1.0 $\mu$l of sample was then analyzed by SDS-polyacrylamide gel electrophoresis with a gradient gel of 10-15% acrylamide (Pharmacia Co.) and an electrophoresis-device (Fast System, Pharmacia Co.). The following molecular weight marker proteins were used to calculate molecular weight : phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.0 kD), and lactalbumin (14.4 kD). After

electrophoresis, protein bands were visualized by silver stain using Phast Silver Stain Kit. The results ~~were~~ are shown in Fig. 4.

A protein band with an apparent molecular weight of 60 KD was detected in the peak 6 ~~protein~~ sample under both reducing and non-reducing conditions. A protein band with an apparent 60 KD was detected under reducing conditions and a protein band with an apparent 120 KD was detected under non-reducing conditions in the peak 7 Sample. ~~protein.~~ Therefore, the protein of peak 7 was considered to be a homodimer of the protein of peak 6.

EXAMPLE 5

Thermostability of OCIF

Twenty $\mu$l of sample from the blue-5PW fractions 51 and 52 was diluted to 30 $\mu$l with 10 mM phosphate buffered saline, pH 7.2, and incubated for 10 min. at 70℃ or 90 ℃, or for 30 min. at 56℃. The heat-treated samples were tested for OCIF activity. The results ~~were~~ are shown in Table 2.

Table 2

Thermostability of OCIF

| Sample | Dilution | | |
|---|---|---|---|
| | 1/300 | 1/900 | 1/2700 |
| untreated | ++ | + | – |
| 70℃, 10 min | + | – | – |
| 56℃, 30 min | + | – | – |
| 90℃, 10 min | – | – | – |

[ ++ means OCIF activity inhibiting osteoclast development more than 80%, +means OCIF activity inhibiting osteoclast development between 30% and 80%, and - means no OCIF activity.]

EXAMPLE 6

Internal amino acid sequence of OCIF protein

Each 2 fractions (1 ml) from fractions 51 to 70 of the blue-5PW fractions were ~~No. 51-70 of blue-5PW fraction was~~ acidified with 10 μl of 25% TFA, and ~~was~~ applied to a reverse phase C4 column (BU-300, 2.1x220mm, manufactured by Perkin-Elmer Co.) equilibrated with 25% ~~of~~ acetonitrile containing 0.1 % TFA. The adsorbed protein was eluted with a 12 ml linear gradient of 25 to 55% acetonitrile at a flow rate of 0.2 ml/min, and the protein fractions corresponding to Peaks 6 and ~~peak 6 and peak~~ 7 were collected, respectively. The protein from ~~of~~ each peak was applied to a protein sequencer (PROCISE 494, Perkin-Elmer Co.). However, the N-terminal sequence of the proteins ~~protein~~ of each peak could not be analyzed. Therefore, the N-terminus ~~N-terminal~~ of the protein of each peak was considered to be blocked. ~~So,~~ Internal ~~internal~~ amino acid sequences of these proteins were therefore analyzed.

The protein from ~~of~~ peak 6 or 7 ~~peak 7~~ purified by C4-HPLC, was concentrated by centrifugation and pyridilethylated under reducing conditions. Briefly, 50 μl of 0.5 M Tris-HCl, pH 8.5, containing 100μg of dithiothreitol, 10mM EDTA, 7 M guanidine-HCl, and 1% CHAPS was added to each of the samples, and the mixtures were ~~mixture was~~ incubated overnight in the dark at ~~a~~ room temperature. Each ~~the~~ mixture was acidified with 25% TFA (a final concentration 0.1%) and ~~was~~ applied to a reverse ~~reversed~~ phase C4 column (BU-300, 2.1x30mm, Perkin-Elmer Co.) equilibrated with 20 % acetonitrile containing 0.1 % TFA. The pyridil-ethylated OCIF

protein was eluted with a 9 ml linear gradient from 20 to 50% acetonitrile at a flow rate of 0.3 ml/min, and each protein peak was collected. The pyridil-ethylated ~~ethyrated~~ OCIF protein was concentrated under vacuum ~~,~~ and dissolved in 25 $\mu$l of 0.1 M Tris-HCl, pH 9, containing 8 M Urea, and 0.1 % Tween 80. Seventy three $\mu$l of 0.1 M Tris-HCl, pH 9, and 0.02 $\mu$g of lysyl endopeptidase (Wako Pure Chemical, Japan) were added to the tube, and incubated at 37 ℃ for 15 hours. Each digest was acidified with 1 $\mu$l of 25% TFA and was applied to a reverse phase C8 column (RP-300, 2.1x220mm, Perkin-Elmer Co.) equilibrated with 0.1% TFA.

The peptide fragments were eluted from the column with a linear gradient of ~~from~~ 0 to 50 % acetonitrile at a flow rate of 0.2 ml/min for 70 min., and each peptide peak was collected. Each peptide fragment (P1 - P3) was applied to the protein sequencer. The sequences of the peptides are ~~were~~ shown in SEQ. ID Nos. 1-3 ~~Sequence Numbers 1 - 3~~, respectively.

EXAMPLE 7

Determination of the nucleotide sequence of OCIF cDNA ~~the OCIF cDNA~~

i) Isolation of poly(A) + RNA from IMR-90 cells

About 10 $\mu$g ~~ug~~ of poly(A) + RNA was isolated from $1x10^8$ cells of IMR-90 ~~by~~ using a Fast Track mRNA isolation kit (Invitrogen) according to the manufacturer's instructions.

ii) Preparation of mixed primers

The following two mixed primers were synthesized based on the amino acid

SEQ. ID Nos.

sequences of two peptides (peptide P2 and peptide P3, [sequence numbers] 2 and 3, respectively). All the oligonucleotides in the mixed primers No. 2F (SEQ. ID No. 107) can code for the amino acid sequence from the sixth residue, glutamine (Gln) to the twelfth residue, leucine (Leu), in peptide P2. All the oligonucleotides in the mixed primers No. 3R (SEQ. ID No. 108) can code for the amino acid sequence from the sixth residue, histidine (His), to the twelfth residue, lysine (Lys), in peptide P3. The sequences of the mixed primers No. 2F and No. 3R were shown in Table 3.

Table 3

No. 2F (SEQ. ID No. 107)

```
5'-CAAGAACAAA CTTTTCAATT-3'
       G  G  G    C  C  GC
                  A
                  G
```

No. 3R (SEQ. ID No. 108)

```
5'-TTTATACATT GTAAAAGAAT G-3'
     C  G      C   G  GCTG
               A      C
               G      T
```

iii) Amplification of an OCIF cDNA fragment by PCR (Polymerase chain reaction)

First strand cDNA was generated using a Superscript II cDNA synthesis kit

(Gibco BRL) and 1 ~~ug~~ µg of poly(A) + RNA obtained in the example 7-i), according to the manufacturer's instructions. The DNA fragment encoding OCIF was obtained by PCR using ~~the~~ cDNA template and the primers shown in EXAMPLE 7-ii).

PCR was performed ~~with the conditions as follows;~~ using the following conditions:

| | | |
|---|---|---|
| 10X Ex Taq Buffer (Takara Shuzo) | 5 | ~~ul~~ µl |
| 2.5 mM solution of dNTPs | 4 | ~~ul~~ µl |
| cDNA solution | 1 | ~~ul~~ µl |
| Ex Taq (Takara Shuzo) | 0.25 | ~~ul~~ µl |
| sterile distilled water | 29.75 | ~~ul~~ µl |
| 40 ~~uM~~ µM solution of primers No. 2F | 5 | ~~ul~~ µl |
| 40 ~~uM~~ µM solution of primers No. 3R | 5 | ~~ul~~ µl |

The components of the reaction were mixed in a microcentrifuge tube. An initial denaturation step at 95 ℃ for 3 min was followed by 30 cycles of denaturation at 95℃ for 30 ~~sec annealing~~ sec, annealing at 50 ℃ for 30 sec and extention at 70 ℃ for 2min. After the amplification, a final extention step was performed at 70 ℃ for 5min. The ~~size of~~ sizes of the PCR products were determined on a 1.5 % agarose gel electrophoresis. ~~About~~ An approximately 400 bp OCIF DNA fragment was obtained.

EXAMPLE 8

Cloning of the OCIF cDNA fragment amplified by PCR and determination of its DNA sequence

The OCIF cDNA fragment amplified by PCR in EXAMPLE 7-iii) was inserted into the plasmid in the plasmid, pBluescript II SK$^-$ using a DNA ligation kit ver. 2 (Takara Shuzo) according to the method of by Marchuk, D. et al. (Nucleic Acids Res., vol 19, p1154, 1991). E.coli strain DH5 $\alpha$ (Gibco BRL) was transformed with the ligation mixture. The transformants were grown and a plasmid containing the OCIF cDNA (about 400 bp) was purified using the commonly used methods. method. This plasmid was called pBSOCIF. The sequence of the OCIF cDNA in pBSOCIF was determined using a Taq Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer). The size of the OCIF cDNA is 397 bp. The OCIF cDNA encodes an amino acid sequence containing 132 residues. The amino acid sequences of the internal peptides (peptide P2 and peptide P3, SEQ. ID Nos. sequence number 2 and 3, respectively) that were used to design the primers were found at the amino or carboxyl terminus of the 132 N- or C- terninal side in the amino acid sequence of the 132 amino acid polypeptide predicted by the 397 bp OCIF cDNA. In addition, the amino acid sequence of the internal peptide P1 (SEQ. ID No. sequence number 1) was also found in the predicted amino acid sequence of the polypeptide. OCIF These data show that the 397 bp OCIF cDNA is a portion of the full length OCIF cDNA.

EXAMPLE 9

Preparation of the DNA probe

The 397 bp OCIF cDNA was prepared according to the conditions described in EXAMPLE 7-iii). The OCIF cDNA was subjected to a preparative agarose gel electrophoresis. The OCIF cDNA was purified from the gel using a QIAEX gel extraction kit (QIAGEN), labeled with [$\alpha$ $^{32}$P]dCTP using or Megaprime DNA labeling

system (Amersham) and used to select a phage containing the full length OCIF cDNA.

EXAMPLE 10

Preparation of the cDNA library

cDNA was generated using a Great Lengths cDNA synthesis kit (Clontech), oligo (dT) primer, [$\alpha$ $^{32}$P]dCTP and 2.5 ~~ug~~ μg of poly(A) + RNA obtained in the example 7-i), according to the manufacturer's instructions. An EcoRI-SalI-NotI adaptor was ligated to the cDNA. The cDNA was separated from ~~the~~ free adaptor DNA and unincorporated free [$\alpha$ $^{32}$P]dCTP. The purified cDNA was precipitated with ethanol and dissolved in 10 ul of TE buffer (10 mMTris-HCl (pH8.0), 1 mM EDTA). The cDNA comprising the adaptor was ligated into ~~with the adaptor was inserted in~~ $\lambda$ZAP EXPRESS vector (Stratagene) at the EcoRI site. The recombinant $\lambda$ZAP EXPRESS phage DNA containing the cDNA was in vitro packaged using a Gigapack gold II packaging extract (Stratagene) ~~and~~, yielding a recombinant $\lambda$ZAP EXPRESS phage library ~~was prepared~~.

EXAMPLE 11

Screening of recombinant phage

Recombinant phages obtained in EXAMPLE 10 were used to infect E. coli strain ~~infected to E. Coli~~, XL1-Blue MRF' (Stratagene) at 37 ℃ for 15 min.. The infected E. coli cells were added to NZY medium containing 0.7 % agar at 50℃ and plated onto ~~on the~~ NZY agar plates. After the plates were incubated at 37 ℃ overnight, Hybond N membranes ~~(Amersham)~~ were placed on the surface of the plates containing plaques. The membranes were denatured in ~~the~~ alkali solution, neutralized, and washed in

Standard methods. onto
2xSSC according to the standard protocol. The phage DNA was immobilized on the
membranes using UV Crosslink (Stratagene). The membranes were incubated in the
hybridization buffer (Amersham) containing 100 $\mu$g/ml salmon sperm DNA at 65℃
for 4 hours and then incubated at 65 ℃ overnight in the same ~~buffer~~ containing
of
$2x10^5$ cpm/ml denatured OCIF DNA probe. The membranes were washed twice with
2xSSC and twice with a solution containing 0.1xSSC and 0.1 % SDS at 65 ℃ for
10 min each time. The positive clones were purified by repeating the screening
a
twice. The purified $\lambda$ZAP EXPRESS phage clone containing about 1.6 kb DNA
of about 1.6Kb
insert was used in the experiments described below. This phage was called
was used to infect E. coli strain
$\lambda$OCIF. The purified $\lambda$OCIF and the infected into E. Coli XL1-Blue MRF'
the in the
(Stratagene) according to a protocol of $\lambda$ZAP EXPRESS cloning kit
(Stratagene). The culture broth of infected XL1-Blue MRF' was prepared.
$\lambda$OCIF
Purified 1OCIF and ExAssist helper phage (Stratagene) were co-infected into
E. coli strain XL-1 blue MRF' according to the protocol supplied with the kit.
The culture broth of the co-infected XL-1 blue MRF' was added to a culture of
E. coli strain XLOR (Stratagene) to transform them. Thus we obtained a
Kanamycin-resistant transformant harboring a plasmid designated pBKOCIF which
is a pBKCMV (Stratagene) vector containing the 1.6 kb insert fragment.

The transformant including the plasmid containing about 1.6 kb OCIF cDNA was
lifting
obtained by picking up the kanamycin-resistant colonies. The plasmid was
in the
called pBKOCIF. The transformant has been deposited to National Institute of
Bioscience and Human-Technology (NIBH), Agency of Industrial Science and
Tecnology as "FERM BP-5267" as pBK/01F10. A national deposit (Accession number,
transferred
FERM P-14998) was transfered to the international deposit, on October 25, 1995

according to the Budapest treaty. The transformant pBK/01F10 was grown and the plasmid pBKOCIF was purified according to the standard protocol. standard methods.

EXAMPLE 12

Determination of the nucleotide sequence of OCIF cDNA containing the full coding region.

The nucleotide sequence of OCIF cDNA obtained in EXAMPLE 11 was determined using a Taq Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer). The primers used were T3, T7 primers (Stratagene) and synthetic primers designed according to the OCIF cDNA sequence. The sequences of these primers are shown in SEQ. ID Nos. sequence numbers 16 to 29. The nucleotide sequence of the OCIF cDNA is shown in SEQ. ID No. sequence number 6 and the amino acid sequence predicted by the cDNA sequence is shown in SEQ. ID No. sequence number 5.

EXAMPLE 13

Production of recombinant OCIF by 293/EBNA cells

i) Construction of the plasmid for expressing OCIF cDNA

pBKOCIF, containing about 1.6 kb OCIF cDNA, was prepared as described in EXAMPLE 11, and digested with restriction enzymes, BamHI and XhoI. The OCIF cDNA insert was cut out, separated by an agarose gel electrophoresis, and purified using a QIAEX gel extraction kit (QIAGEN). The purified OCIF cDNA insert was ligated into the insert was ligated using DNA ligation kit ver. 2 (Takara Shuzo) to the expression vector pCEP4 (Invitrogen) using DNA ligation Kit ver. 2 (Takara Shuzo) digested with restriction enzymes, BamHI and XhoI. E. Coli Strain E. coli. DH5$\alpha$ (Gibco BRL) was transformed with the ligation mixture.

The transformants were grown and the plasmid containing the OCIF cDNA (about 1.6 kb) was purified using a QIAGEN column (QIAGEN). The expression plasmid pCEPOCIF was precipitated with ethanol~~,~~ and dissolved in sterile distilled water ~~was used in the exprerimentss~~ for use in the experiments described below.

ii) Transient expression of OCIF cDNA and analysis of ~~the~~ OCIF biological activity

Recombinant OCIF was produced using the expression plasmid~~,~~ pCEPOCIF (prepared in EXAMPLE 13-i) according to the method described below. $8x10^5$ cells of 293/EBNA (Invitrogen) were inoculated ~~in~~ into each well of ~~the~~ a 6-well plate using IMDM containing 10 % fetal calf serum (Gibco BRL). After the cells were incubated for 24 hours, the culture medium was removed and the cells were washed with serum free IMDM. The expression plasmid~~,~~ pCEPOCIF and lipofectamine (Gibco BRL) were diluted with OPTI-MEM (Gibco BRL), ~~and were~~ mixed, and added to the cells in each well according to the manufacture's instructions. Three $\mu$g of pCEPOCIF and 12 $\mu$l of lipofectamine were used for each transfection. After the cells were incubated with pCEPOCIF and lipofectamine for 38 hours, the medium was replaced with 1 ml of OPTI-MEM. After ~~the transfected cells were incubated~~ incubation for 30 hours, the conditioned medium was harvested and used for the biological assay. The biological activity of OCIF was analysed according to the method described below. Bone marrow cells obtained from ~~mice, 17 days-old,~~ 17 day old mice were suspended in $\alpha$-MEM (manufactured by GIBCO BRL Co.) containing 10% FBS, ~~$2x10^{-8}M$~~ $1x10^{-8}M$ activated vitamin ~~$D_3$, and each~~ $D_3$ and a test sample, and were ~~inoculatd~~ inoculated and cultured for 7 days at 37℃ in humidified 5%$CO_2$ as described in EXAMPLE 2. During incubation, 160

$\mu$l of old medium in each well was replaced with the same volume of the fresh medium containing test sample diluted with $1\times10^{-8}$M of activated vitamin $D_3$ and $\alpha$-MEM containing FBS on day 3 and day 5. On day 7, after washing the wells with phosphate buffered saline, cells were fixed with ethanol/acetone (1:1) for 1 min. and ~~then~~ osteoclast development was tested using an acid phosphatase activity mesuring kit (Acid Phosphatase, Leucocyte, Catalog No. 387-A, Sigma Co.). ~~The decrease of~~ A decrease in the number of TRAP positive cells was taken as an OCIF activity. ~~As result, the~~ The conditioned medium showed the same OCIF activity as natural OCIF protein from IMR-90 conditioned medium (Table 4).

Table 4

OCIF activity of 293/EBNA conditioned medium.

| Cultured Cell | Dilution | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| OCIF expression vector transfected | ++ | ++ | ++ | ++ | ++ | + | - |
| vector transfected | - | - | - | - | - | - | - |
| untreated | - | - | - | - | - | - | - |

[ ++ ; OCIF activity inhibiting osteoclast development more than 80%, + ; OCIF activity inhibiting osteoclast development between 30% and 80%, and - ; no

OCIF activity. ]

iii) Isolation of recombinant OCIF protein from 293/EBNA-conditioned medium

293/EBNA-conditioned medium (1.8 l) obtained by cultivating the cells described in example 13-ii) was supplemented with 0.1 % ~~of~~ CHAPS and filtrated ~~with~~ using a 0.22 $\mu$m membrane filter (Steribecs GS, ~~Milipore~~ Millipore Co.). The conditioned medium was applied to ~~50 ml of a~~ a 50 ml heparin Sepharose CL-6B column (2.6 x 10 cm, Pharmacia Co.) equilibrated with 10mM Tris-HCl, pH 7.5. After washing the column with 10mM Tris-HCl, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 4 ml/min for 100 min. and 8 ml fractions ~~(8 ml)~~ were collected. Using 150 $\mu$l of each fraction, OCIF activity was assayed according to the method described in EXAMPLE 2. An OCIF active 112 ml fraction, ~~OCIF active fraction (112 ml)~~ eluted with approximately 0.6 to 1.2 M NaCl, was obtained.

One hundred twelve ml of the active fraction was diluted to 1000 ml with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5, and applied to a heparin affinity column (heparin-5PW, 0.8 x 7.5 cm, Tosoh Co.) equilibrated with 10mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 10mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 0.5ml/min for 60 min. ~~,~~ and 0.5 ml fractions ~~(0.5 ml)~~ were collected. Four $\mu$l of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described in EXAMPLE 4. ~~On SDS-PAGE under reducing conditions, a~~ A single band of rOCIF protein with an apparent 60 ~~KD~~ kD was detected in fractions from 30 to 32 ~~, under~~ by SDS-PAGE under reducing conditions.

Bands apparent molecular weights of kD

non-reducing conditions, bands of rOCIF protein with an apparent 60 KD and

kD under non-reducing conditions

120 KD were also detected in fractions from 30 to 32. The isolated rOCIF

from fractions

fraction from 30 to 32 was designated as recombinant OCIF derived from 293/EBNA (rOCIF(E)). 1.5 ml of the rOCIF(E) (535 $\mu$g/ml) was obtained when determined by the method of Lowry, using bovine serum albumin as a standard protein.

## EXAMPLE 14

### Production of recombinant OCIF using CHO cells

i) Construction of the plasmid for expressing OCIF

pBKOCIF containing about 1.6 kb OCIF cDNA was prepared as described in EXAMPLE 11, and digested with restriction enzymes, SalI and EcoRV. About 1.4 kb OCIF cDNA insert was separated by an agarose gel electrophoresis, and

a

purified from the gel using QIAEX gel extraction kit (QIAGEN). The expression vector, pcDL-SR $\alpha$296 (Molecular and Cellular Biology, vol 8, p466, 1988) was digested with restriction enzymes, PstI and KpnI. About 3.4 kb of the expression vector fragment was cut out, separated by agarose gel

a

electrophoresis, and purified from the gel using QIAEX gel extraction kit (QIAGEN). The ends of the purified OCIF cDNA insert and the expression vector

a

fragment were blunted using DNA blunting kit (Takara Shuzo). The purified OCIF

a

cDNA insert and the expression vector fragment were ligated using DNA ligation

coli strain

kit ver. 2 (Takara Shuzo). E. coli. DH5a $\alpha$ (Gibco BRL) was transformed with

A

the ligation mixture. The transformant containing the OCIF expression plasmid, pSR$\alpha$OCIF was obtained.

ii) Preparation of the expression plasmid

The transformant containing the OCIF expression plasmid, pSR $\alpha$ OCIF ~~preprared in the~~ prepared in example 13-i) and the transformant containing the mouse DHFR expression plasmid, pBAdDSV shown in WO92/01053 were grown according to ~~the~~ standard ~~method.~~ methods. Both plasmids were purified by alkali treatment, polyethylene glycol precipitation, and cesium ~~chrolide~~ chloride density gradient ultra centrifugation according to the method of Maniatis et al. (Molecular cloning, 2nd edition).

iii) Adaptation of CHOdhFr- cells to the protein free medium

CHOdhFr- cells (ATCC, CRL 9096) were cultured in IMDM containing 10 % fetal calf serum. The cells were adapted to EX-CELL 301 (JRH Biosciecnce) and then adapted to EX-CELL PF CHO (JRH Biosciecnce) according to the manufacture's instructions.

iv) Transfection of the OCIF expression plasmid, and the mouse DHFR expression plasmid, ~~to~~ into CHOdhFr- cells.

CHOdhFr- cells prepared in EXAMPLE 14-iii) were transfected by electroporation with pSR $\alpha$ OCIF and pBAdDSV prepared in EXAMPLE 14-ii). ~~200~~ Two hundred $\mu$g of pSR $\alpha$ OCIF and 20 $\mu$g of pBAdDSV were dissolved under sterile conditions in 0.8 ml of IMDM (Gibco BRL) containing 10 % fetal calf serum~~CO~~. ~~2x10^7 cells of~~ CHOdhFr- cells (2x10^7) were suspended in 0.8 ml of this medium. The cell suspension was transfered to a cuvette (Bio Rad) and the cells were transfected by electroporation using a gene pulser (Bio Rad) under ~~condition~~ the conditions of

360 V and 960 $\mu$F. The suspension of electroporated cells was transferred to T-flasks (Sumitomo Bakelite) containing 10 ml of EX-CELL PF-CHO, and incubated in the $CO_2$ incubator for 2 days. ~~Then the~~ The transfected cells were then inoculated ~~in~~ into each well of a 96 well plate (Sumitomo Bakelite) at a density of 5000 cells/well and cultured for about 2 weeks. The transformants expressing DHFR are selected since EX-CELL PF-CHO does not contain nucleotides and the parental cell line CHO dhFr- can not grow in this medium. Most of the transformants expressing DHFR express OCIF since the OCIF expression plasmid was used ten times as much as the mouse DHFR expression plasmid. The transformants whose conditioned medium had high OCIF activity were selected among the transformants expressing DHFR according to the method described in EXAMPLE 2. The transformants that express large amounts of OCIF were cloned by limiting dilution. The clones whose conditioned medium had high OCIF activity were selected as described above and ~~the~~ a transformant expressing large ~~amount~~ amounts of OCIF named 5561, was obtained.

v) Production of recombinant OCIF

To produce recombinant OCIF (rOCIF), ~~EX-CELL 301 medium (3 l) in a 3 l-spiner flask was inoculated with the clone (5561)~~ clone 5561 was inoculated into a 3l-spiner flask with Ex-CELL 301 medium (3l) at a cell-density of $1x10^5$ cells/ml. The 5561 cells were cultured in a spiner flask at 37℃ for 4 to 5 days. When the concentration of the 5561 cells reached ~~to~~ $1x10^6$ cells/ml, about 2.7 l of the conditioned medium was harvested. Then about 2.7 l of EX-CELL 301 was added to the spiner flask and the 5561 cells were cultured repeatedly. About 20 l of the conditioned medium was harvested using the three spiner

flasks.

vi) Isolation of recombinant OCIF protein from CHO cell-Conditioned [cells-conditioned] medium

CHO cell-Conditioned [CHOcells-conditioned] medium (1.0 l) described in EXAMPLE [EXAMPL] 14-v) was supplemented with 1.0 g [of] CHAPS and filtrated with a 0.22 $\mu$m membrane filter (Steribecks GS, Millipore [Milipore] Co.). The conditioned medium was applied to a heparin Sepharose-FF column (2.6 x 10 cm, Pharmacia Co.) equilibrated with 10 mM Tris-HCl, pH 7.5. After washing the column with 10 mM Tris-HCl, 0.1 % CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 4 ml/min for 100 min. and 8ml fractions [(8 ml)] were collected. Using 150 $\mu$l of each fraction, OCIF activity was assayed according to the method described in EXAMPLE 2. An active [Active] fraction (112 ml) eluted with approximately 0.6 to 1.2 M NaCl was obtained.

The 112 ml [of] active fraction was diluted to 1200 ml with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5, and applied to an [a] affinity column (Blue [blue]-5PW, 0.5 x 5.0 cm, Tosoh Co.) equilibrated with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 3 M NaCl at a flow rate of 0.5ml/min for 60 min.[,] and fractions (0.5 ml) were collected. Four $\mu$l of each fraction were [was] subjected to SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described in EXAMPLE 4. [On SDS-PAGE under reducing conditions,] A [a] single band of rOCIF protein with an apparent molecular weight of 60 KD using SDS-PAGE under reducing Conditions. was detected in fractions 30 to 38[, under non-reducing conditions,] Bands [bands] of rOCIF protein with apparent molecular weights of 60 KD and 120 KD using SDS-PAGE under non-reducing conditions were also detected in fractions

30 to 38. The isolated rOCIF fraction, from fractions 30 to 38, was designated as purified recombinant OCIF derived from CHO cells (rOCIF(C)). 4.5 ml of the rOCIF(C) (113 $\mu$g/ml) was ~~obtained when~~ obtained, as determined by the method of Lowry using bovine serum albumin as a standard protein.

EXAMPLE 15

Determination of N-terminal amino acid sequence of rOCIFs

Each 3 $\mu$g of the isolated rOCIF(E) and rOCIF(C) was adsorbed to polyvinylidene difluoride (PVDF) membranes with Prospin (PERKIN ELMER Co.). The membranes were washed with 20 % ethanol and the N-terminal amino acid sequences of the adsorbed proteins were analyzed by protein sequencer (PROCISE 492, PERKIN ELMER Co.). The determined N-terminal amino acid sequence is shown
SEQ. ID No.
in [sequence No.] 7.

glutamic acid located at position 22
The N-terminal amino acid of rOCIF(E) and rOCIF(C) was [the 22th amino
from Met of the translation start site, as shown in SEQ. ID No.
acid of glutamine from Met as translation starting point, as shown in sequence
number] 5. The 21 amino acids from Met to Gln were identified as a signal peptide. The N-terminal amino acid sequence of OCIF isolated from IMR-90
could not be determined. glutamic acid
conditioned medium [was undetectable.] Accordingly, the N-terminal [glutamine]
the conversion of glutamic acid
of OCIF may be blocked by [converting from] [glutamine] to pyroglutamine within
[culturing or purifing.] cell culture or purification steps.

EXAMPLE 16

Biological activity of recombinant(r) OCIF and natural(n) OCIF

in
i) Inhibition of vitamin $D_3$ induced osteoclast formation [from] murine bone marrow cells

samples were
Each the rOCIF(E) and nOCIF [sample was] diluted with $\alpha$-MEM (GIBCO BRL Co.) containing 10% FBS and $2\times10^{-8}$M of activated vitamin $D_3$ (a final concentration of 250 ng/ml). Each sample was serially diluted with the same
of a
medium, and 100 $\mu$l of each diluted sample was added to each well [in] 96-well

plates. Bone marrow cells obtained from ~~mice, 17 days-old,~~ 17 day old mice were inoculated at a cell density of $3x10^5$ cells/100 $\mu$l/ well ~~to~~ into each well ~~in~~ of a 96-well plat~~es~~ and cultured for 7 days at 37℃ in humidified 5%$CO_2$. On day 7, the cells were fixed and stained with ~~a~~ an acid phosphatase ~~mesuring~~ measuring kit (Acid Phosphatase, Leucocyte, No387-A, Sigma) according to the method described in EXAMPLE 2. ~~The~~ A decrease ~~of~~ in acid phosphatase activity (TRAP) was taken as an indication of OCIF activity. ~~The~~ A decrease ~~of~~ in acid phosphatase-positive cells was evaluated by solubilizing the pigment of dye and measuring absorbance. ~~In detail,~~ Briefly, 100 $\mu$l of a mixture of 0.1 N NaOH and dimethylsulfoxide (1:1) was added to each well and the well was vibrated to solubilize the dye. After solubilizing the dye completely, an absorbance of each well was measured at 590 nm, subtracting the absorbance at 490 nm using a microplate reader (Immunoreader NJ-2000, InterMed). The microplate reader was adjusted to 0 absorbance using a well with monolayered bone marrow cells which ~~was~~ were cultured in the medium without activated vitamin $D_3$. ~~The decrease of~~ A decrease in TRAP activity was expressed as a percentage of the control absorbance value (=100%) ~~of the~~ (the solubilized dye from wells with bone marrow cells ~~which were~~ cultured in the absence of ~~OCIF.~~ OCIF). The results are shown in Table 5.

Table 5

Inhibition of vitamin D3-induced osteoclast formation from murine bone marrow cells

| OCIF concentration(ng/ml) | 250 | 125 | 63 | 31 | 16 | 0 |
|---|---|---|---|---|---|---|
| rOCIF(E) | 0 | 0 | 3 | 62 | 80 | 100 |

| | | | | | | |
|---|---|---|---|---|---|---|
| nOCIF | 0 | 0 | 27 | 27 | 75 | 100 (%) |

Both nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner at concentrations in the concentration of 16 ng/ml or higher greater.

ii) Inhibition of vitamin D3-induced osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

The effect Effect of OCIF on osteoclast formation induced by Vitamin $D_3$ in co-cultures of stromal cells and mouse spleen cells was tested according to the method of N. Udagawa et al. (Endocrinology, vol. 125, p1805-1813, 1989). Briefly, samples of In detail, each of rOCIF(E), rOCIF(C), and nOCIF were sample was serially diluted with α-MEM (GIBCO BRL Co.) containing 10% FBS, $2x10^{-8}$M of activated vitamin $D_3$, and $2x10^{-7}$M dexamethasone, and 100μl of each of the diluted samples was added to each well of in 96 well-microwell plates. Murine bone marrow-derived stromal ST2 cells (RIKEN Cell Bank RCB0224) at ; $5x10^3$ cells per 100μl of α-MEM containing 10% FBS FBS, and spleen cells from 8 week old ddy mice at ddy mice, 8 weeks-old, ; $1x10^5$ cells per 100 μl in the same medium, were inoculated into to each well of a 96-well plate in 96-well plates and cultured for 5 days at 37℃ in humidified 5%$CO_2$. On day 5, the cells were fixed and stained using an with a kit for acid phosphatase kit (Acid Phosphatase, Leucocyte, No387-A, Sigma). A decrease in The decrease of acid phosphatase-positive cells was taken as an indication as OCIF activity. The decrease in of acid phosphatase-positive cells was evaluated according to the method described in EXAMPLE 16-i). The results are shown in Table 6 (rOCIF(E) and rOCIF(C)) and Table 7 (rOCIF(E) and are shown in Table 6 ; rOCIF(E) and rOCIF(C), and Table 7 ; rOCIF(E) and nOCIF. nOCIF).

Table 6

Inhibition of osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

| OCIF concentration(ng/ml) | 50 | 25 | 13 | 6 | 0 | |
|---|---|---|---|---|---|---|
| rOCIF(E) | 3 | 22 | 83 | 80 | 100 | |
| rOCIF(C) | 13 | 19 | 70 | 96 | 100 | (%) |

Table 7

Inhibition of osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

| OCIF concentration(ng/ml) | 250 | 63 | 16 | 0 | |
|---|---|---|---|---|---|
| rOCIF(E) | 7 | 27 | 37 | 100 | |
| rOCIF(C) | 13 | 23 | 40 | 100 | (%) |

nOCIF, rOCIF(E) and rOCIF(C) inhibited osteoclast formation in a dose dependent manner ~~in the concentration~~ at concentrations of 6 - 16 ng/ml or ~~higher~~ greater.

iii) Inhibition of PTH-induced osteoclast formation ~~from~~ in murine bone marrow cells.

~~Effect~~ The effect of OCIF on osteoclast formation induced by PTH was tested according to the method of N. Takahashi et al. (Endocrinology, vol. 122,

Briefly, samples of each of

p1373-1382, 1988). In detail, each the rOCIF(E) and nOCIF sample (125 ng/ml)

were

was serially diluted with $\alpha$-MEM (manufactured by GIBCO BRL Co.) containing

of the wells of

10% FBS and $2x10^{-8}$M PTH, and 100$\mu$l of each the diluted samples was added to

17 day old ddy mice

96 well-plates. Bone marrow cells from ddy mice, 17 days-old, at a cell

density of $3x10^5$ cells per 100$\mu$l of $\alpha$-MEM containing 10% FBS were

into of a plate

inoculated to each well in 96-wells plates and cultured for 5 days at 37℃ in

acetone

humidified 5%$CO_2$. On day 5, the cells were fixed with ethanol/aceton (1:1) for

an Kit

1 min. at room temperature and stained with a kit for acid phosphatase (Acid Phosphatase, Leucocyte, No387-A, Sigma) according to the method described in

A in an indication of

EXAMPLE 2. The decrease of acid phosphatase-positive cells was taken as OCIF

in

activity. The decrease of acid phosphatase-positive cells was evaluated according to the method described in EXAMPLE 16-i). The results are shown in Table 8.

Table 8

Inhibition of PTH-induced osteoclast formation from murine bone marrow cells.

| OCIF concentration(ng/ml) | 125 | 63 | 31 | 16 | 8 | 0 |
|---|---|---|---|---|---|---|
| rOCIF(E) | 6 | 58 | 58 | 53 | 88 | 100 |
| nOCIF | 18 | 47 | 53 | 56 | 91 | 100 |

nOCIF and rOCIF(E) inhibited osteoclast formation in a dose

at concentrations

dependent manner in the concentration of 16 ng/ml or higher greater.

iv) Inhibition of IL-11-induced osteoclast formation

The effect
Effect of OCIF on osteoclast formation induced by IL-11 was tested according to the method of T. Tamura et al. (Proc. Natl. Acad. Sci. USA, vol.
Briefly, samples of each of
90, p11924-11928, 1993). In detail, each rOCIF(E) and nOCIF sample was were
serially diluted with $\alpha$-MEM (GIBCO BRL Co.) containing 10% FBS and 20 ng/ml
a
IL-11 and 100 $\mu$l of each the diluted sample was added to each well in 96-well
plate.
plates. Newborn mouse calvaria-derived pre-adipocyte MC3T3-G2/PA6 cells (RIKEN
at
Cell Bank RCB1127) ; $5\times10^3$ cells per 100 $\mu$l of $\alpha$-MEM containing 10% FBS, and
8 week old ddy mouse, at
spleen cells from ddy mouse, 8 weeks-old, ; $1\times10^5$ cells per 100 $\mu$l in the
into of a plate
same medium, were inoculated to each well in 96-well plates and cultured for
5 days at 37 ℃ in humidified 5% $CO_2$. On day 5, the cells were fixed and
an kit
stained with a kit for acid phosphatase (Acid Phosphatase, Leucocyte, No387-A,
a
Sigma). Acid phosphatase positive cells were counted under microscope and a
an indication of
decrease of the cell numbers was taken as OCIF activity. The results are shown in Table 9.

Table 9

| OCIF concentration(ng/ml) | 500 | 125 | 31 | 7.8 | 2.0 | 0.5 | 0 |
|---|---|---|---|---|---|---|---|
| nOCIF | 0 | 0 | 1 | 4 | 13 | 49 | 31 |
| rOCIF(E) | 0 | 0 | 1 | 3 | 10 | 37 | 31 |

Both nOCIF and rOCIF(E) inhibited osteoclast formation in a dose
at concentrations
dependent manner in the concentration of 2 ng/ml or higher greater.

The results shown in Table 4-8 indicated that OCIF inhibits all the

vitamin $D_3$, PTH, and IL-11-induced osteoclast formations at almost the same doses. Accordingly, OCIF could would be able to be used for treatment of the treating different types of bone disorders with due to decreased bone mass, which that are caused by different substances which that induce bone resorption.

## EXAMPLE 17

### Isolation of monomer-type OCIF and dimer-type OCIF

Each rOCIF(E) and rOCIF(C) sample containing 100 $\mu$g of OCIF protein, was supplemented with 1/100 volume of 25 % trifluoro acetic acid and applied to a reverse phase column (PROTEIN-RP, 2.0x250 mm, YMC Co.) equilibrated with 30 % acetonitrile containing 0.1 % trifluoro acetic acid. OCIF protein was eluted from the column with a linear gradient from 30 to 55 % acetonitrile at a flow rate of 0.2 ml/min for 50 min. and each OCIF peak was collected. Each the The monomer-type OCIF peak fraction and dimer-type OCIF peak fraction was were lyophilized, respectively. each lyophilized.

## EXAMPLE 18

### Determination of the molecular weight of recombinant OCIFs

Each 1 $\mu$g of the isolated monomer-type and dimer-type nOCIF purified using a reverse phase column according to EXAMPLE 3-iv) and each 1 $\mu$g of monomer-type and dimer-type rOCIF described in EXAMPLE 17 was concentrated under vaccum, respectively vacuum. Each sample was incubated in the buffer for SDS-PAGE, subjected to SDS-polyacrylamide gel electrophoresis, and protein bands on the gel were stained with silver according to the method described in

EXAMPLE 4. Results of electrophoresis under non-reducing conditions and reducing conditions are shown in Figure 6 and Figure 7. Figures 6 and 7, respectively.

A protein band with an apparent molecular weight of 60 KD was detected in each monomer-type OCIF sample, and a protein band with an apparent molecular weight of 120 KD was detected in each dimer-type OCIF sample in under non-reducing conditions. A protein band with an apparent molecular weight of 60 KD was detected in each monomer-type OCIF sample under reducing conditions. Accordingly, the molecular weights of monomer-type nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells and rOCIF from CHO cells were almost the same (60kD). Molecular weights of dimer-type nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells, and rOCIF from CHO cells were also the same (120KD).

EXAMPLE 19

Removal of the Remove N-linked Oligosaccharide chain and measuring the Mesuring molecular weight of natural and recombinant OCIF

Each sample containing 5μg of the isolated monomer-type and dimer-type nOCIF purified using a reverse phase column according to EXAMPLE 3-iv) and each sample containing 5 μg of monomer-type and dimer-type rOCIF described in EXAMPLE 17 were concentrated under vaccum. Each sample was dissolved in 9.5 μl of 50 mM sodium phosphate buffer, pH 8.6, containing 100 mM 2-mercaptoethanol, supplemented with 0.5 μl of 250 U/ml N-glycanase (Seikagaku kogyo Co.) and incubated for one day at 37 ℃. Each sample was supplemented with 10 μl of 20 mM Tris-HCl, pH 8.0 containing 2 mM EDTA, 5 % SDS, and 0.02 % bromo-phenol blue and heated for 5 min at 100 ℃. Each 1 μl

of the samples was subjected to SDS-polyacrylamide gel electrophoresis, and protein bands on the gel were stained with silver as described in EXAMPLE 4. The patterns of electrophoresis are shown in Figure 8.

An apparent molecular weight of each of the deglycosylated nOCIF from IMR-90 cells, rOCIF from CHO cells, and rOCIF from 293/EBNA cells was 40 KD under reducing conditions. An apparent molecular weight of each of the untreated nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells, and rOCIF from CHO cells was 60 KD under reducing conditions. Accordingly, the results indicate that the OCIF proteins are glycoproteins with N-linked sugar chains.

EXAMPLE 20

Cloning of OCIF variant cDNAs and determination of their DNA [squences] sequences

The plasmid pBKOCIF, comprising [which is inserted] OCIF cDNA into plasmid [to] pBKCMV (Stratagene), was obtained [from one of some purified positive phage] as in example 10 and 11. Further [And more], during the screening of the cDNA library with the 397 bp OCIF cDNA probe, the transformants containing plasmids whose insert sizes were different from that of pBKOCIF were obtained. These transformants containing the plasmids were grown and the plasmids were purified according to the standard method. The sequence of the insert DNA in each plasmid was determined using a Taq Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer). The [used primers] primers used were T3, T7 [primers] (Stratagene) and synthetic primers prepared based on the nucleotide sequence of OCIF cDNA. There are four OCIF variants (OCIF2, 3, 4, and 5) in addition to OCIF. The nucleotide sequence of OCIF2 is shown in SEQ. ID No. [the sequence number] 8 and the amino acid sequence of OCIF 2 predicted by the nucleotide sequence is shown in SEQ. ID No. [the sequence number] 9. The nucleotide sequence of OCIF3 is shown in SEQ. ID No. [the sequence number] 10 and the amino acid sequence of OCIF3 predicted by the nucleotide sequence is shown in SEQ. ID No. [the sequence number] 11. The nucleotide sequence of OCIF4 is shown in SEQ. ID No. [the sequence number] 12 and the amino acid sequence of OCIF4 predicted by the nucleotide sequence is shown in SEQ. ID No. [the sequence number] 13. The nucleotide sequence of OCIF5 is shown in SEQ. ID No. [the sequence number] 14 and the amino acid sequence of OCIF5 predicted by the nucleotide sequence is shown in SEQ. ID No. [the sequence number] 15. The structures of OCIF variants are shown in Figures 9 to 12 and are briefly described [in brief] below.

OCIF2

The OCIF2 cDNA has a deletion of 21 bp from guanine at nucleotide number 265 to guanine at nucleotide number 285 in the OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6). Accordingly, OCIF2 has a deletion of 7 amino acids from glutamic acid (Glu) at amino acid number 68 to glutamine (Gln) at amino acid number 74 in OCIF (SEQ. ID No. ~~sequence number~~ 5).

OCIF3

The OCIF3 cDNA has a point mutation at nucleotide number 9 in the OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6) where cytidine is replaced with guanine. Accordingly, OCIF3 has a mutation where ~~and~~ asparagine (Asn) at amino acid number -19 in OCIF (SEQ. ID No. ~~sequence number~~ 5) is replaced with lysine (Lys). The mutation seems to be located in the signal sequence and has ~~have~~ no essential effect on the secretion of ~~secreted~~ OCIF3. OCIF3 cDNA has a deletion of 117 bp from guanine at nucleotide number 872 to cytidine at nucleotide number 988 in the OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6).

Accordingly, OCIF3 has a deletion of 39 amino acids from threonine (Thr) at amino acid number 270 to leucine (Leu) at amino acid number 308 in OCIF (SEQ. ID No. ~~sequence number~~ 5).

OCIF4

The OCIF4 cDNA has two point mutations in the OCIF cDNA (SEQ. ID No. 6).

~~OCIF4 cDNA has two point mutations in OCIF cDNA (sequence number 6).~~ Cytidine at nucleotide number 9 is replaced with guanine and guanine at nucleotide number 22 is replaced with thymidine in the OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6).

Accordingly, OCIF4 has two mutations. Asparagine (Asn) at amino acid number -19 in OCIF (SEQ. ID No. ~~sequence number~~ 5) is replaced with lysine (Lys), and alanine (Ala)

at amino acid number −14 is replaced with serine (Ser). These mutations seem to be located in the signal sequence and have no essential effect on the secreted OCIF4.

OCIF4 cDNA has about 4 kb DNA, comprising ~~which is the~~ intron 2 of the OCIF gene, inserted between nucleotide number 400 and nucleotide number 401 in the OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6). The open reading frame stops in intron 2.

Accordingly, OCIF4 has an additional novel amino acid sequence containing 21 amino acids after alanine (Ala) at amino acid number 112 in OCIF (SEQ. ID No. 5) ~~sequence number 5).~~

OCIF5

The OCIF5 cDNA has a point mutation at nucleotide number 9 in the OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6) where cytidine is replaced with guanine.

Accordingly, OCIF5 has a mutation where ~~and~~ asparagine (Asn) at amino acid number −19 in OCIF (SEQ. ID No. ~~sequence number~~ 5) is replaced with lysine (Lys). The mutation seems to be located in the signal sequence and has ~~have~~ no essential effect on the secretion of ~~secreted~~ OCIF5.

The OCIF5 cDNA has the latter portion (about 1.8 kb) of intron 2 between nucleotide number 400 and nucleotide number 401 in OCIF cDNA (SEQ. ID No. ~~sequence number~~ 6). The open reading frame stops in the latter portion of intron 2.

Accordingly, OCIF5 has an additional novel amino acid sequence containing 12 amino acids after alanine (Ala) at amino acid number 112 in OCIF (SEQ. ID No. 5) ~~sequence number 5).~~

EXAMPLE 21

Production of OCIF variants

i) Construction of the plasmid for expressing OCIF variants

~~The plasmid~~ Plasmids containing OCIF2 or OCIF3 cDNA ~~was~~ were obtained as described in EXAMPLE 20 and called pBKOCIF2 and pBKOCIF3, respectively. pBKOCIF2 and pBKOCIF3 were digested with restriction enzymes~~,~~ BamHI and XhoI. The OCIF2 and OCIF3 cDNA inserts were separated by agarose gel electrophoresis~~,~~ and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The purified OCIF2 and OCIF3 cDNA inserts were individually ligated using a DNA ligation kit ver. 2 (Takara Shuzo) to the expression vector pCEP4 (Invitrogen) that had been digested with restriction enzymes~~,~~ BamHI and XhoI. E. ~~coli~~ coli strain DH5 $\alpha$ (Gibco BRL) was transformed with the ligation mixture.

The plasmid containing OCIF4 cDNA was obtained as described in EXAMPLE 20 and called pBKOCIF4. pBKOCIF4 was digested with restriction enzymes~~,~~ SpeI and XhoI (Takara Shuzo). The OCIF4 cDNA insert was separated by ~~an~~ agarose gel electrophoresis, and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The purified OCIF4 cDNA insert was ligated using a DNA ligation kit ver. 2 (Takara Shuzo) to ~~the~~ an expression vector pCEP4 (Invitrogen) that had been digested with restriction enzymes~~,~~ NheI and XhoI (Takara Shuzo). E. ~~coli.~~ coli strain DH5 $\alpha$ (Gibco BRL) was transformed with the ligation mixture.

The plasmid containing OCIF5 cDNA was obtained as described in EXAMPLE 20 and ~~was~~ called pBKOCIF5. pBKOCIF5 was digested with the restriction enzyme~~,~~ HindIII (Takara Shuzo). The 5' portion of the coding region in the OCIF5 cDNA insert was separated by agarose gel electrophoresis~~,~~ and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The OCIF expression plasmid, pCEPOCIF,

obtained in EXAMPLE 13-i) was digested with the restriction enzyme, HindIII (Takara Shuzo). The 5' portion of the coding region in the OCIF cDNA was removed. The rest of the plasmid that contains pCEP vector and the 3' portion of the coding region of OCIF cDNA was called pCEPOCIF-3'. pCEPOCIF-3' was separated by an agarose gel electrophoresis, and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The OCIF5 cDNA HindIII fragment and pCEPOCIF-3' were ligated using a DNA ligation kit ver. 2 (Takara Shuzo). E. coli strain coli. DH5 α (Gibco BRL) was transformed with the ligation mixture.

The transformants obtained obtained transformants were grown at 37 ℃ overnight and the OCIF variant variants expression plasmids (pCEPOCIF2, pCEPOCIF3, pCEPOCIF4, and pCEPOCIF5) were purified using QIAGEN columns column (QIAGEN). These OCIF-variant variants-expression plasmids were precipitated with ethanol, dissolved in sterile distilled water, and used in the experiments exprerimants described below.

ii) Transient expression of OCIF variant cDNAs and analysis of the biological activity of recombinant OCIF variants.

Recombinant OCIF variants were produced using the expression plasmids, plasmid pCEPOCIF2, pCEPOCIF3, pCEPOCIF4, and pCEPOCIF5 prepared as described in EXAMPLE 21-i) according to the method described in EXAMPLE 13-ii). The biological activities of recombinant OCIF variants were analysed. The results were that these OCIF variants (OCIF2, OCIF3, OCIF4, and OCIF5) had a weak activity.

EXAMPLE 22

Preparation of OCIF mutants

i) Construction of a plasmid vector for subcloning cDNAs encoding OCIF mutants

The plasmid vector ($5\ \mu g$) described in EXAMPLE 11 was digested with restriction enzymes Bam HI and Xho I ( Takara Shuzo). The digested DNA was subjected to a preparative agarose gel electrophoresis. A DNA fragment with an approximate size of 1.6 kilobase pairs (kb) that contained the entire coding sequence for OCIF was purified from the gel using a QIAEX gel extraction kit (QIAGEN). The purified DNA was dissolved in $20\ \mu l$ of sterile distilled water. This solution was designated DNA solution 1. p Bluescript II SK + ($3\ \mu g$) (Stratagene) was digested with restriction enzymes Bam HI and Xho I (Takara Shuzo). The digested DNA was subjected to preparative agarose gel electrophoresis. A DNA fragment with an approximate size of 3.0 kb was purified from the gel using a QIAEX DNA extraction kit (QIAGEN). The purified DNA was dissolved in $20\ \mu l$ of sterile distilled water. The this solution was designated DNA solution 2. One microliter of DNA solution 2, $4\ \mu l$ of DNA solution 1 and $5\ \mu l$ of ligation buffer I from a of DNA ligation kit ver. 2 (Takara Shuzo) were mixed and incubated at 16 ℃ for 30 min. (The ligation mixture was used for in the transformation of E. coli in a manner described below). Conditions for transformation of E. coli were as follows. One hundred microliters of competent E. coli strain DH5 α cells (GIBCO BRL) and $5\ \mu l$ of the ligation mixture were was mixed in a sterile 15-ml tube (IWAKI glass). The tube was kept on ice for 30 min. After incubation for 45 sec at 42℃, to the cells was added 250 $\mu l$ of L broth (1% Tryptone, 0.5% yeast extract, 1% NaCl) was added to the cells. The cell suspension was then incubated for 1hr. at 37℃ with shaking. Fifty

microliters of the cell suspension was plated onto an L-agar plate containing 50 $\mu$g/ml of ampicillin. The plate was incubated overnight at 37℃.

Six colonies which grew on the plate were individually each incubated in 2 ml each of L-broth containing 50 $\mu$g/ml of ampicillin overnight at 37℃ with shaking. The structure of the plasmids in the colonies was analyzed. A plasmid in which the 1.6-kb DNA fragment containing the entire OCIF cDNA is inserted between the digestion sites of Bam HI and Xho I of pBluescript II SK + was obtained and designated as pSK + -OCIF.

ii) Preparation of mutants in which one of the Cys residues in OCIF is replaced with a Ser residue

1) Introduction of mutations into OCIF cDNA

OCIF mutants were prepared in which one of the five Cys residues present in OCIF at positions 174, 181, 256, 298 and 379 (in SEQUENCE NO 4) was replaced with a Ser residue and were designated OCIF-C19S(174Cys to Ser), OCIF-C20S (181Cys to Ser), OCIF-C21S (256Cys to Ser), OCIF-C22S (298Cys to Ser) and OCIF-C23S (379Cys to Ser), respectively.

To prepare the mutants, nucleotides encoding the corresponding Cys residues were replaced with those encoding Ser. Mutagenesis was carried out by a two-step polymerase chain reaction (PCR). The first step of the PCRs consisted of two reactions, PCR 1 and PCR 2.

| | | | |
|---|---|---|---|
| PCR 1 | 10X Ex Taq Buffer (Takara Shuzo) | 10 | $\mu$l |
| | 2.5 mM solution of dNTPs | 8 | $\mu$l |
| | the plasmid vector described in EXAMPLE 11 (8ng/ml) | 2 | $\mu$l |
| | sterile distilled water | 73.5 | $\mu$l |

| | | |
|---|---|---|
| | 20 $\mu$M solution of primer 1 | 5 $\mu$l |
| | 100 $\mu$M solution of primer 2 (for mutagenesis) | 1 $\mu$l |
| | Ex Taq (Takara Shuzo) | 0.5 $\mu$l |
| PCR 2 | 10X Ex Taq Buffer (Takara Shuzo) | 10 $\mu$l |
| | 2.5 mM solution of dNTPs | 8 $\mu$l |
| | the plasmid vector described in EXAMPLE 11 (8ng/ml) | 2 $\mu$l |
| | sterile distilled water | 73.5 $\mu$l |
| | 20 $\mu$M solution of primer 3 | 5 $\mu$l |
| | 100 $\mu$M solution of primer 4 (for mutagenesis) | 1 $\mu$l |
| | Ex Taq (Takara Shuzo) | 0.5 $\mu$l |

Specific sets of primers were used for each mutation and other components were unchanged. Primers used for the reactions are shown in Table 10. The nucleotide sequences of the primers are shown in ~~SEQUENCE NO:~~ SEQ. ID Nos. 20, 23, 27 and 30–40. The PCRs were performed under the following conditions ~~as follows~~. An initial denaturation step at 97℃ for 3 min was followed by 25 cycles of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 3 min. After these amplification cycles, final extension was performed at 70℃ for 5 min. The ~~size~~ sizes of the PCR ~~prodcts was~~ products were confirmed by agarose gel electrophoresis ~~using~~ of the reaction ~~solution~~ solutions. After the first PCR, excess primers were removed using an Amicon microcon (Amicon). The final volume of the solutions that contained the PCR products were made to 50 $\mu$l with sterile distilled water. These purified PCR products were used for the second PCR (PCR 3).

| | | |
|---|---|---|
| PCR 3 | 10X Ex Taq Buffer (Takara Shuzo) | 10 $\mu$l |

| | |
|---|---|
| 2.5 mM solution of dNTPs | 8 μl |
| solution containing DNA fragment obtained from PCR 1 | 5 μl |
| solution containing DNA fragment obtained from PCR 2 | 5 μl |
| sterile distilled water | 61.5 μl |
| 20 μM solution of primer 1 | 5 μl |
| 20 μM solution of primer 3 | 5 μl |
| Ex Taq (Takara Shuzo) | 0.5 μl |

Table 10

| mutants | primer-1 | primer-2 | primer-3 | primer-4 |
|---|---|---|---|---|
| OCIF-C19S | IF 10 | C19SR | IF 3 | C19SF |
| OCIF-C20S | IF 10 | C20SR | IF 3 | C20SF |
| OCIF-C21S | IF 10 | C21SR | IF 3 | C21SF |
| OCIF-C22S | IF 10 | C22SR | IF 14 | C22SF |
| OCIF-C23S | IF 6 | C23SR | IF 14 | C23SF |

The reaction conditions were exactly the same as those for PCR 1 or PCR 2. The ~~size~~ sizes of the PCR ~~prodcts was~~ products were confirmed by 1.0 % or 1.5 % agarose gel electrophoresis. The DNA fragments were precipitated with ethanol, dried under vacuum and dissolved in 40 μl of sterile distilled water. The solutions containing DNA fragments with ~~mutation~~ mutations C19S, C20S, C21S, C22S and C23S were

designated as DNA solution A, DNA solution B, DNA solution C, DNA solution D and DNA solution E, respectively.

The DNA fragment which is contained in solution A (20 μl) was digested with restriction enzymes Nde I and Sph I (Takara Shuzo). A DNA fragment with an approximate size of 400 base pairs (bp) was extracted from a preparative agarose gel and dissolved in 20 μl of sterile distilled water. This DNA solution was designated DNA solution 3. Two micrograms of pSK + −OCIF was were digested with restriction enzymes Nde I and Sph I. A DNA fragment with an approximate size of 4.2 kb was purified from a preparative agarose gel with using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated as DNA solution 4. Two microliters of DNA solution 3, 3 μl of DNA solution 4 and 5 μl of ligation buffer I of from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5α E. coli DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C19S.

The DNA fragment which is contained in solution B (20 μl) was digested with restriction enzymes Nde I and Sph I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel with using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated DNA solution 5. Two microliters of DNA solution 5, 3 μl of DNA solution 4 and 5 μl of ligation buffer I of from a DNA ligation kit

ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C20S.

The DNA fragment which is contained in solution C (20 $\mu$l) was digested with restriction enzymes Nde I and Sph I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel using a with QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 6. Two microliters of DNA solution 6, 3 $\mu$l of DNA solution 4 and 5$\mu$l of ligation buffer I from a of DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C21S.

The DNA fragment which is contained in solution D (20 $\mu$l) was digested with restriction enzymes Nde I and Bst PI. A DNA fragment with an approximate size of 600 bp was extracted from a preparative agarose gel using a with QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 7. Two micrograms of pSK + -OCIF was were digested with restriction enzymes Nde I and Bst PI. A DNA fragment with an approximate size of 4.0 kb was extracted from a preparative agarose gel with using a QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled

water. This DNA solution was designated [as] DNA solution 8. Two microliters of DNA solution 7, 3 $\mu$l of DNA solution 8 and 5$\mu$l of ligation buffer I [of] from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA in which the 600-bp Nde I-BstPI fragment with the mutation (the C22S mutation) is substituted for the 600-bp Nde I-Bst PI fragment of pSK+ -OCIF by analyzing the DNA structure. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C22S.

The DNA fragment which is contained in solution E (20 $\mu$l) was digested with restriction enzymes Bst PI and Eco RV. A DNA fragment with an approximate size of 120 bp was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled water. This DNA solution was designated [as] DNA solution 9. Two micrograms of pSK + -OCIF [was] were digested with restriction enzymes Bst EII and Eco RV. A DNA fragment with an approximate size of 4.5 kb was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled water. This DNA solution was designated [as] DNA solution 10. Two microliters of DNA solution 9, 3 $\mu$l of DNA solution 10 and 5 $\mu$l of ligation buffer I [of] from a DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5$\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by

DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C23S.

2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-C19S, pSK-OCIF-C20S, pSK-OCIF-C21S, pSK-OCIF-C22S and pSK-OCIF-C23S were digested with restriction enzymes Bam HI and Xho I. The 1.6 kb Bam HI-Xho I DNA fragment encoding each OCIF mutant was isolated and dissolved in 20 $\mu$l of sterile distilled water. The DNA solutions that contain 1.6 kb cDNA fragments derived from pSK-OCIF-C19S, pSK-OCIF-C20S, pSK-OCIF-C21S, pSK-OCIF-C22S and pSK-OCIF-C23S were designated C19S DNA solution, C20S DNA solution, C21S DNA solution, C22S DNA solution and C23S DNA solution, respectively. Five micrograms of a expression vector pCEP 4 (Invitrogen) was were digested with restriction enzymes Bam HI and Xho I. A DNA fragment with an approximate size of 10 kb was purified and dissolved in 40 $\mu$l of sterile distilled water. This DNA solution was designated as pCEP 4 DNA solution. One microliter of pCEP 4 DNA solution and 6 $\mu$l of either C19SDNA C19S DNA solution, C20S DNA solution, C21S DNA solution, C22S DNA solution or C23S DNA solution were independently mixed with 7 $\mu$l of ligation buffer I of from a DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent E. coli strain DH5$\alpha$ cells (100 $\mu$l) were transformed with 7 $\mu$l of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmid in which a 1.6-kb cDNA fragment is inserted between the recognition sites of Bam HI and Xho I of pCEP 4 by analyzing the DNA structure. The plasmide plasmids which were obtained containing the cDNA encoding OCIF-C19S, OCIF-C20S, OCIF-C21S, OCIF-C22S and OCIF-C23S were designated pCEP4-OCIF-C19S, pCEP4-OCIF-C20S, pCEP4-OCIF-C21S, pCEP4-OCIF-C22S and

pCEP4-OCIF-C23S, respectively.

ii) Preparation of domain-deletion mutants of OCIF

(1) deletion mutagenesis of OCIF cDNA

A series of OCIF mutants with deletions ~~of~~ from Thr 2 to Ala 42, from Pro 43 to Cys 84, from Glu 85 to Lys 122, from Arg 123 to Cys 164, from Asp 177 to Gln 251 ~~and~~ or from Ile 252 to His 326 were prepared (positions of the amino acid residues are shown in ~~SEQUENCE NO~~ SEQ. ID No. 4). These mutants were designated as OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2, respectively.

Mutagenesis was performed by two-step PCR as described in EXAMPLE 22-(ii). The primer sets for the reactions are shown in Table 11 and the nucleotide sequences of the primers are shown in ~~SEQUENCE NO~~ SEQ. ID Nos. 19, 25, 40-53, and 54.

Table 11

| mutants | primer-1 | primer-2 | primer-3 | primer-4 |
|---|---|---|---|---|
| OCIF-DCR1 | XhoI F | DCR1R | IF 2 | DCR1F |
| OCIF-DCR2 | XhoI F | DCR2R | IF 2 | DCR2F |
| OCIF-DCR3 | XhoI F | DCR3R | IF 2 | DCR3F |
| OCIF-DCR4 | XhoI F | DCR4R | IF 16 | DCR4F |
| OCIF-DDD1 | IF 8 | DDD1R | IF 14 | DDD1F |
| OCIF-DDD2 | IF 8 | DDD2R | IF 14 | DDD2F |

The final PCR products were precipitated with ethanol, dried under vacuum and dissolved in 40 $\mu$l of sterile distilled water. Solutions of DNA [fragment] fragments coding for portions of OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2 were designated [as] DNA solutions F, G, H, I, J and K, respectively.

The DNA fragment [which is] contained in solution F (20 $\mu$l) was digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated DNA solution 11. Two micrograms of pSK+ -OCIF [was] were digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 4.0 kb was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated DNA solution 12. Two microliters of DNA solution 11, 3 $\mu$l of DNA solution 12 and 5 $\mu$l of ligation buffer I [of] from a DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR1.

The DNA fragment which is contained in solution G (20 $\mu$l) was digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA

solution was designated as DNA solution 13. Two microliters of DNA solution 13, 3 $\mu$l of DNA solution 12 and 5 $\mu$l of ligation buffer I from a of DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent E. coli strain DH5a cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid a DNA. DNA . DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR2.

The DNA fragment which is contained in solution H (20 $\mu$l) was digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel using a with QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 14. Two microliters of DNA solution 14, 3 $\mu$l of DNA solution 12 and 5 $\mu$l of ligation buffer I from a of DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR3.

The DNA fragment which is contained in solution I (20 $\mu$l) was digested with restriction enzymes Xho I and Sph I. A DNA fragment with an approximate size of 900 bp was extracted from a preparative agarose gel using a with QIAEX gel

extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated [as] DNA solution 15. Two micrograms of pSK+ -OCIF [was] were digested with restriction enzymes Xho I and Sph I. A DNA fragment with an approximate size of 3.6 kb was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated [as] DNA solution 16. Two microliters of DNA solution 15, 3 $\mu$l of DNA solution 16 and 5 $\mu$l of ligation buffer I from a [of] DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR4.

The DNA fragment [which is] contained in solution J (20 $\mu$l) was digested with restriction enzymes BstP I and Nde I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated [as] DNA solution 17. Two microliters of DNA solution 17, 3 $\mu$l of DNA solution 8 and 5 $\mu$l of ligation buffer I from a [of] DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DDD1.

The DNA fragment which is contained in solution K (20 $\mu$l) was digested with restriction enzymes Nde I and BstP I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel using a with QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 18. Two microliters of DNA solution 18, 3 $\mu$l of DNA solution 8 and 5$\mu$l of ligation buffer I from a of DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DDD2.

2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-DCR1, pSK-OCIF-DCR2, pSK-OCIF-DCR3, pSK-OCIF-DCR4, pSK-OCIF-DDD1 and pSK-OCIF-DDD2 were digested with restriction enzymes Bam HI and Xho I. The Bam HI-Xho I DNA fragment containing the entire coding sequence for each OCIF mutant was isolated and dissolved in 20 $\mu$l of sterile distilled water. These DNA solutions that contain the Bam HI-Xho I fragment derived from pSK-OCIF-DCR1, pSK-OCIF-DCR2, pSK-OCIF-DCR3, pSK-OCIF-DCR4, pSK-OCIF-DDD1 and pSK-OCIF-DDD2 were designated DCR1 DNA solution, DCR2 DNA solution, DCR3 DNA solution, DCR4 DNA solution, DDD1 DNA solution and DDD2 DNA solution, respectively. One microliter of pCEP 4 DNA solution and 6$\mu$l of either DCR1 DNA solution, DCR2 DNA solution, DCR3 DNA solution, DCR4 DNA solution, DDD1 DNA solution or DDD2 DNA solution were independently mixed with 7$\mu$l of

ligation buffer I [of] from a DNA ligation kit ver. 2 and ligation the reactions were carried out. Competent E. coli strain DH5$\alpha$ cells (100 $\mu$l) were transformed with 7 $\mu$l of each ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA in which the DNA fragment with deletions is inserted between the recognition sites of Bam HI and Xho I of pCEP 4 by analyzing the DNA structure. The plasmids containing the cDNA encoding OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2 were designated [as] pCEP4-OCIF-DCR1, pCEP4-OCIF-DCR2, pCEP4-OCIF-DCR3, pCEP4-OCIF-DCR4, pCEP4-OCIF-DDD1 and pCEP4-OCIF-DDD2, respectively.

iii) Preparation of OCIF with C-terminal domain truncation

(1) mutagenesis of OCIF cDNA

A series of OCIF mutants with deletions [of] from Cys at amino acid residue 379 to Leu 380, from Ser 331 to Leu 380, from Asp 252 to Leu 380, from Asp 177 to Leu 380, from Arg 123 to Leu 380 and from Cys 86 to Leu 380 was prepared. Positions of the amino acid residues are shown in SEQ. ID No. [SEQUENCE NO:] 4. These mutants were designated as OCIF-CL, OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3, respectively.

Mutagenesis for OCIF-CL was performed by the two-step PCR as described in EXAMPLE 22-(ii). The primer set for the reaction is shown in Table 12. The nucleotide sequences of the primers are shown in SEQ. ID Nos. [SEQUENCE NO] 23, 40, 55, and 56. The final PCR products were precipitated with ethanol, dried under vacuum and dissolved in 40$\mu$l of sterile distilled water. This DNA solution was designated [as] solution L.

The DNA fragment which is contained in solution L (20 $\mu$l) was digested with restriction enzymes BstP I and EcoR V. A DNA fragment with an approximate size of 100 bp was extracted from a preparative agarose gel using a with QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 19. Two microliters of DNA solution 19, 3 $\mu$l of DNA solution 10 (described in EXAMPLE 22-(ii)) and 5$\mu$l of ligation buffer I from a of DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5$\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-CL. Mutagenesis of OCIF cDNA to prepare OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3 was performed by a one-step PCR reaction.

PCR reactions for mutagenesis to prepare OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3 were as follows:

| | |
|---|---|
| 10X Ex Taq Buffer (Takara Shuzo) | 10 $\mu$l |
| 2.5 mM solution of dNTPs | 8 $\mu$l |
| the plasmid vector containing the entire OCIF cDNA described in EXAMPLE 11 (8ng/ml) | 2 $\mu$l |
| sterile distilled water | 73.5 $\mu$l |
| 20 $\mu$M solution of primer OCIF Xho F | 5 $\mu$l |
| 100 $\mu$M solution of primer (for mutagenesis) | 1 $\mu$l |
| Ex Taq (Takara Shuzo) | 0.5 $\mu$l |

Table 12

| mutants | primer-1 | primer-2 | primer-3 | primer-4 |
|---|---|---|---|---|
| OCIF-CL | IF 6 | CL R | IF 14 | CL F |

Specific primers were used for each mutagenesis and other components were unchanged.

Primers used for the mutagenesis are shown in Table 13. Their nucleotide sequences are shown in SEQ. ID No. SEQUENCE NO:57-61. The components of each PCR were mixed in a microcentrifuge tube and PCR was performed as follows. The microcentrifuge tubes were treated for 3 minutes at 97 ℃ and then incubated sequentially, for 30 seconds at 95 ℃, 30 seconds at 50 ℃ and 3 minutes at 70 ℃. This three-step incubation procedure was repeated 25 times, and after that, the tubes were incubated for 5 minutes at 70 ℃. An aliquot of the reaction mixture was removed from each tube and analyzed by an agarose gel electrophoresis to confirm the size of each product.

The size of the PCR products was confirmed on an agarose gel. Excess primers in the PCRs were removed using an Amicon microcon (Amicon) after completion of the reaction. The DNA fragments were precipitated with ethanol, dried under vacuum and dissolved in 40 $\mu$l of sterile distilled water. The DNA fragment in each DNA solution was digested with restriction enzymes Xho I and Bam HI. After the reactions, DNA was precipitated with ethanol, dried under vacuum and dissolved in 20 $\mu$l of sterile distilled water.

The solutions containing the DNA fragment with the CC deletion, the CDD2

deletion, the CDD1 deletion, the CCR4 deletion and the CCR3 deletion were designated ~~as~~ CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution and CCR3 ~~CC R3~~ DNA solution, respectively.

Table 13

| mutants | primers for the mutagenesis |
|---|---|
| OCIF-CC | CC R |
| OCIF-CDD2 | CDD2 R |
| OCIF-CDD1 | CDD1 R |
| OCIF-CCR4 | CCR4 R |
| OCIF-CCR3 | CCR3 R |

(2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-CL was digested with restriction enzymes Bam HI and Xho I. The Bam HI-Xho I DNA fragment containing the entire coding sequence for OCIF-CL was isolated and ~~isolatedand~~ dissolved in 20 $\mu l$ of sterile distilled water. This DNA solution was designated ~~as~~ CL DNA solution. One microliter of pCEP 4 DNA solution and 6 $\mu l$ of either ~~of~~ CL DNA solution, CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution or CCR3 DNA solution were independently mixed with 7 $\mu l$ of ligation buffer I from a ~~of~~ DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent E. coli strain DH5$\alpha$ cells (100 $\mu l$) were transformed with 7 $\mu l$ of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmids which have the

desirable mutations in the OCIF cDNA by analyzing the DNA structure. In each plasmid, the OCIF cDNA fragment having a deletion ~~were~~ was inserted between the recognition sites of Xho I and Bam HI of pCEP 4. The plasmids containing the cDNA encoding OCIF-CL, OCIF-CC, OCIF-CDD1, OCIF-CDD2, OCIF-CCR4 and OCIF-CCR3 were designated pCEP4-OCIF-CL, pCEP4-OCIF-CC, pCEP4-OCIF-CDD2, pCEP4-OCIF-CDD1, pCEP4-OCIF-CCR4 and pCEP4-OCIF-CCR3, respectively.

iv) Preparation of OCIF mutants with C-terminal ~~truncation~~ truncations

(1) Introduction of C-terminal ~~truncation~~ truncations to OCIF

A series of OCIF mutants with C-terminal ~~truncation~~ truncations was prepared. An OCIF mutant in which 10 residues ~~of~~ from Gln at 371 to Leu at 380 ~~are~~ were replaced with 2 residues ~~of Leu-Val~~ (Leu-Val) was designated OCIF-CBst. An OCIF mutant in which 83 residues ~~of~~ from Cys 298 to Leu 380 ~~are~~ were replaced with 3 residues ~~of Ser-Leu-Asp~~ (Ser-Leu-Asp) was designated OCIF-CSph. An OCIF mutant in which 214 residues ~~of~~ from Asn 167 to Leu 380 ~~are~~ were removed was designated OCIF-CBsp. ~~OCIF muatant~~ An OCIF mutant in which 319 residues ~~of~~ from Asp 62 to Leu 380 ~~are~~ were replaced with 2 residues ~~of Leu-Val~~ (Leu-Val) was designated OCIF-CPst. Positions of the amino acid residues are shown in ~~SEQUENCE NO:~~ SEQ. ID No. 4.

Two micrograms each of pSK + -OCIF ~~was~~ were digested with ~~one of the restriction enzymes,~~ Bst PI, Sph I, PstI (Takara Shuzo)~~, and~~ or Bsp EI (New England Biolabs)~~, and~~ followed by phenol extraction and ethanol precipitation. The precipitated DNA was dissolved in 10 $\mu$l of sterile distilled water. ~~Ends~~ The ends of the DNAs in 2 $\mu$l of each solution were blunted using a DNA blunting kit in a final ~~volumes~~ volume of 5 $\mu$l. To the reaction mixtures, 1 $\mu$g (1 $\mu$l) of an Amber

codon-containing Xba I linker (5'-CTAGTCTAGACTAG-3') and 6 $\mu$l of ligation buffer I ~~of~~ from a DNA ligation kit ver. 2 were added.

After the ligation reactions, 6 $\mu$l each of the reaction mixtures was used to transform E. coli strain DH5$\alpha$. Ampicillin-resistant transformants were screened for clones containing plasmids. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmids thus obtained were named pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp and pSK-OCIF-CPst, respectively.

(2) Construction of vectors ~~for~~ expressing the OCIF mutants

pSK-OCIF-CBst, ~~pSK-OCIF- CSph~~ pSK-OCIF-CSph, pSK-OCIF-CBsp and pSK-OCIF-CPst were digested with restriction enzymes Bam HI and Xho I. The 1.5 kb ~~of~~ DNA fragment containing the entire coding sequence for each OCIF mutant was isolated and dissolved in 20 $\mu$l of sterile distilled water. These DNA solutions that ~~contain~~ contained the Bam HI-XhoI fragment derived from pSK-OCIF-CBst, ~~pSK-OCIF- CSph~~ pSK-OCIF-CSph, pSK-OCIF-CBsp ~~and~~ or pSK-OCIF-CPst were designated ~~as~~ CBst DNA solution, CSph DNA solution, CBsp DNA solution and CPst DNA solution, respectively. One microliter of pCEP 4 DNA solution (described in EXAMPLE 22-ii)) and 6 $\mu$l of either CBst DNA solution, CSph DNA solution, CBsp DNA solution or CPst DNA solution were independently mixed with 7 $\mu$l of ligation buffer I ~~of~~ from a DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent E. coli strain DH5$\alpha$ cells (100 $\mu$l) were transformed with 7 $\mu$l of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmids in which the cDNA fragment ~~is~~ was inserted between the recognition sites of Bam HI and Xho I of pCEP 4 by analyzing the DNA structure. The plasmids containing the cDNA encoding OCIF-CBst, OCIF-CSph, OCIF-CBsp ~~and~~ or OCIF-CPst

were designated as pCEP4-OCIF-CBst, pCEP4-OCIF- CSph, pCEP4-OCIF-CBsp and pCEP4-OCIF-CPst, respectively.

Preparation

v) Prepareti on of vectors for expressing the OCIF mutants

a

E. coli clones harboring the expression vectors for OCIF mutants (total of 21

using columns

clones) were grown and the vectors were purified by QIAGEN column (QIAGEN). All the expression vectors were precipitated with ethanol and dissolved in appropriate volumes of sterile distilled water and used for further

manipulations

manipupations shown below.

vi) Transient expression of the cDNAs for OCIF mutants and biological activities of the mutants

OCIF mutants were produced using the expression vectors prepared in EXAMPLE 22-v). The method was essentially the same as described in EXAMPLE 13. Only the modified points are described below. A 24-well plate was used for the DNA transfection. 2X10[5] cells of 293/EBNA suspended in IMDM containing 10% fetal

a 24 well

bovine serum were seeded into each well of the plate. One microgram of purified vector DNA and 4μl of lipofectamine were used for each

A mixture of the

transfection. Mixture of an expression vector and lipofectamine in OPTI-MEM (GIBCO BRL) in a final volume of 0.5 ml was added to the cells in a well. After

5% $CO_2$

the cells were incubated at 37℃ for 24 hr in a $CO_2$ incubator, the medium was replaced with 0.5 ml of Ex-cell 301 medium (JSR). The cells were incubated

a further 48 5% $CO_2$

at 37 ℃ for 48 more hours in the $CO_2$ incubator. The conditioned medium was

in assays

collected and used for assay for in vitro biological activity. The nucleotide

SEQ. ID Nos.

sequences of cDNAs for the OCIF mutants are shown in SEQUENCE NO:83-103. The

SEQ. ID Nos.

deduced amino acid sequences for the OCIF mutants are shown in SEQUENCE NO:

62-82. The assay for in vitro biological activity was performed as described in EXAMPLE 13. The Antigen ~~Antigen~~ concentration of each conditioned medium was determined by ELISA as described in EXAMPLE 24. Table 14 shows ~~specific~~ the activity of each mutant ~~the mutants~~ relative to that of the unaltered OCIF.

Table 14

| mutants | activity |
|---|---|
| the unaltered OIF | ++ |
| OCIF-C19S | + |
| OCIF-C20S | ± |
| OCIF-C21S | ± |
| OCIF-C22S | + |
| OCIF-C23S | ++ |
| OCIF-DCR1 | ± |
| OCIF-DCR2 | ± |
| OCIF-DCR3 | ± |
| OCIF-DCR4 | ± |
| OCIF-DDD1 | + |
| OCIF-DDD2 | ± |
| OCIF-CL | ++ |
| OCIF-CC | ++ |
| OCIF-CDD2 | ++ |
| OCIF-CDD1 | + |
| OCIF-CCR4 | ± |
| OCIF-CCR3 | ± |
| OCIF-CBst | ++ |

| | |
|---|---|
| OCIF-CSph | ++ |
| OCIF-CBsp | ± |
| OCIF-CPst | ± |

++ indicates relative activity more than 50% of that of the unaltered OCIF; + indicates relative activity between 10% and 50% ± indicates relative; activity less than 10%, or production level too low to determine the accurate biological activity.

vii) ~~western~~ Western blot analysis

Ten microliters of the final conditioned medium was used for western blot analysis. Ten microliters of ~~the~~ each sample were mixed with 10 μl of SDS-PAGE sample buffer (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 20 μg/ml ~~bromo phenol~~ bromophenol blue, pH 6.8), boiled for 3 min. and subjected to ~~a~~ 10 % SDS ~~polyacryl amide~~ polyacrylamide gel electrophoresis under non-reducing conditions. After the electrophoresis, the separated proteins were blotted to PVDF membrane (ProBlott®, Perkin Elmer) using a semi-dry electroblotter (BIO-RAD). The membrane was incubated at 37℃ with horseradish ~~peroxidase labeled~~ peroxidase-labeled anti-OCIF antibodies for 2 hr. After the membrane was washed, protein bands which react with the labeled antibodies were detected using an ECL system (Amersham). Two protein bands with approximate molecular masses of 60kD and 120kD were detected for the unaltered OCIF. On the other hand, almost exclusively a 60kD protein band was detected for the OCIF-C23S, OCIF-CL and OCIF CC mutants. Protein bands with ~~an~~ approximate masses of 40kD-50kD and 30kD-40kD were the major ones for OCIF-CDD2 and OCIF-CDD1, respectively. These results indicate that Cys at 379 is responsible for the

dimer formation, both the monomers and the dimers maintain the biological activity and a deletion of residues from Asp at 177 to Leu at 380 does not abolish the biological activity of OCIF (positions of the amino acid ~~resare~~ residues are shown in ~~SEQUENCE NO:~~ SEQ. ID NO. 4).

EXAMPLE 23

## Isolation of human genomic OCIF gene

### i) Screening of a human genomic library

An amplified human placenta genomic library in Lambda FIX II vector (Stratagene) purchased from STRATAGENE was screened for the gene encoding human OCIF using the human OCIF cDNA as a probe. Essentially, screening was done according to the instruction manual supplied with the genomic library. The basic protocols described in Molecular Cloning: A Laboratory Manual were also also were employed to manipulate phage, E. coli, and DNA .

The library was titered, and $1x10^6$ pfu of phage was mixed with XL1-Blue MRA host E. coli cells and plated onto on 20 plates (9 cm x 13 cm) with 9 ml per plate of top agarose. The plates were incubated overnight at 37℃. Filter plaque lifts were prepared using Hybond-N nylon membranes (Amersham). The membranes were processed by denaturation in a solution containing 1.5 M NaCl and 0.5 M NaOH for 1 minute at room temperature. The membranes were then neutralized by placing each one successively for one minute each in 1 M Tris-HCl (pH7.5) and a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) successfully for one minute each. The membranes were then transferred onto a filter paper wetted wet with 2xSSC. Phage DNA was fixed onto on the membranes with 1200 μ Joules of UV energy using a in STRATALINKER UV crosslinker 2400 (STRATAGENE) and the membranes were air dried. The membranes were immersed in Rapid Hybridization buffer (Amersham) and incubated for one hour at 65 ℃ before hybridization with $^{32}P$-labeled cDNA probe in the same buffer overnight at 65℃. Screening probe was prepared by labeling the

OCIF cDNA with $^{32}$P using the Megaprime DNA labeling system (Amersham). Approximately, $5x10^5$cpm probe was used for each ml of hybridization buffer. After the hybridization, the membranes were rinsed in 2xSSC for five minutes at room temperature. The membranes were then washed four times, 20 minutes each time, in 0.5xSSC containing 0.1 % SDS at 65 ℃. After the final wash, the membranes were dried and subjected to autoradiography at -80 ℃ with SUPER HR-H X-ray film (FUJI ~~PFOTO~~ PHOTO FILM Co., Ltd.) and an intensifying screen. Upon examination of the autoradiograms, six positive signals were detected. Agar plugs were picked from the regions corresponded to these signals for phage purification. Each agar plug was soaked overnight in 0.5 ml of SM buffer containing 1% chloroform to extract phage. Each extract containing phage was diluted 1000 fold with SM buffer and an aliquot of ~~1 ml or 20 ml~~ 1μl or 20μl was mixed with host E. coli described above. The mixture was plated ~~on~~ onto agar plates with top agarose as described above. The plates were incubated overnight at 37 ℃, and filter lifts were prepared, prehybridized, hybridized, washed and autoradiographed as described above. This process of phage purification was applied to all six positive signals initially detected on the autoradiograms and was repeated until all phage plaques on agar plates hybridize with the cDNA probe. After purification, agar plugs of each phage isolate were soaked in SM buffer containing 1% chloroform and stored at 4 ℃. Six individual phage isolates were designated λOIF3, λOIF8, λOIF9, λOIF11, λOIF12 and λOIF17, respectively.

ii) Analysis of the genomic clones by restriction enzyme digestion and

Southern blot hybridization

DNA was prepared from each phage isolate by the plate lysate method as described in <u>Molecular Cloning: A Laboratory Manual</u>. DNA prepared from each phage was digested with restriction enzymes and the fragments derived from the digestion were separated on agarose gels. The fragments were then transferred to nylon membranes and subjected to Southern blot hybridization using OCIF cDNA as a probe. The results of the analysis revealed that the six phage isolates are individual clones. Among these fragments derived from ~~the~~ restriction enzyme digestion, those fragments which hybridized with the OCIF cDNA probe were subcloned into plasmid vectors and subjected to ~~the~~ nucleotide sequence analysis as described below.

iii) Subcloning restriction fragments derived from genomic clones into plasmid vectors and determining their ~~determination of the~~ nucleotide sequence.

$\lambda$OIF8 DNA was digested with restriction enzymes EcoRI and NotI, and the DNA fragments derived therefrom ~~these from~~ were separated on a 0.7% agarose gel. The 5.8 kilobase pair ~~pairs~~ (kb) EcoRI/NotI fragment was extracted from the gel using a QIAEX II Gel Extraction Kit (QIAGEN) according to the procedure recommended by the manufacturer. The 5.8 kb EcoRI/NotI fragment was ligated with pBluescript II SK+ vector (STRATAGENE), which had been linearized with restriction enzymes EcoRI and NotI, using Ready-To-Go T4 DNA Ligase (Pharmacia) according to the procedure recommended by the manufacturer. Competent DH5 $\alpha$ E. coli cells (Amersham) were transformed with the recombinant plasmid and transformants were selected on L-plates containing 50 $\mu$g/ml of ampicillin.

A clone harboring the recombinant plasmid containing the 5.8 kb EcoRI/NotI fragment was isolated and this plasmid was termed pBSG8-5.8. pBSG8-5.8 was digested with HindIII and a 0.9 kb of DNA fragment derived from this digestion was isolated in the same manner as described above. This 0.9 kb fragment was then cloned into in pBluescript II SK- at the HindIII site as described above. This recombinant plasmid containing 0.9 kb HindIII fragment was denoted pBS8H0.9.

λOIF11 DNA was digested with EcoRI and 6 kb, 3.6 kb, 2.6 kb EcoRI fragments were isolated in the same manner as described above and cloned in into a pBluescript II SK+ vector at the EcoRI site as described above. These recombinant plasmids were termed pBSG11-6, pBSG11-3.6, and pBSG11-2.6, respectively. pBSG11-6 was digested with HindIII and the digest was separated applied on a 0.7 % agarose gel. Three fragments, 2.2 kb, 1.1 kb, and 1.05 kb in length, were extracted from the gel and cloned independently into in pBluescript II SK- vector at the HindIII site in the same manner as described above. These recombinant plasmids were termed pBS6H2.2, pBS6H1.1 pBS6 H1.1 and pBS6H1.05, respectively.

The nucleotide sequence of the cloned genomic DNA was determined using a ABI Dyedeoxy Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER) and a 373A DNA Sequencing system (Applied Biosystems). Plasmids pBSG8-5.8, pBS8H0.9, pBSG11-6, pBSG11-3.6, pBSG11-2.6, pBS6H2.2, pBS6H1.1 and pBS6H1.05 were prepared according to the alkaline-SDS procedure as described in Molecular Cloning: A Laboratory Manual and used as templates for the DNA sequence analysis. The nucleotide Nucleotide sequence of the human OCIF gene is was presented in SEQ. ID No. Sequence No 104 and SEQ. ID No. Sequence No 105. The nucleotide sequence of the DNA,

between exon 1 and exon 2 was not entirely determined. There is a stretch of approximately 17 kb ~~of nucleotides~~ between the sequences given in SEQ. ID ~~sequence~~ No. 104 and SEQ. ID ~~sequence~~ No. 105.

## EXAMPLE 24

### Quantitation of OCIF by EIA

i) Preparation of anti-OCIF antibody

Male JW rabbits (Kitayama LABES Co., LTD) weighing 2.5-3.0 kg were used in ~~for~~ immunization for preparing antisera. ~~Three male JW rabbits (Kitayama LABES Co., LTD) weighing 2.5-3.0 kg were used for immunization.~~ For immunization, an emulsion was prepared by mixing an equal volume of rOCIF (200 $\mu$g/ml) and complete Freund's adjuvant (Difco, Cat. 0638-60-7). Three ~~The~~ rabbits were immunized subcutaneously six times at ~~the interval of~~ one week intervals with 1 ml of emulsion per injection. ~~The rabbits were injected six times at the interval of seven days subcutaneously.~~ Whole blood was obtained ten days after the final immunization and serum was isolated. ~~separated.~~ Antibody was purified from serum as follows. Antiserum was diluted two-fold with PBS. After adding ammonium sulfate at a final concentration of 40% w/v, the ~~40 w/v %,~~ antiserum was allowed to stand at 4 ℃ for 1 hr. The precipitate ~~Precipitate~~ obtained by centrifugation at 8000 x g for 20 min. was dissolved in a small volume of PBS and was dialyzed against PBS. The resultant ~~resulting~~ solution was loaded onto a Protein G-Sepharose column (Pharmacia). After washing with PBS, absorbed immunoglobulin G was eluted with 0.1 M glycine-HCL buffer (pH 3.0). The eluate was immediately ~~Elutes were~~ neutralized with 1.5 M Tris-HCL buffer (pH 8.7) ~~immediately~~ and and ~~were~~ dialyzed against PBS. Protein

concentration was determined by absorbance at 280nm ($E^{1\%}$ 13.5).

Horseradish ~~peroxidase labeled~~ peroxidase-labeled antibody was prepared using an ImmunoPure Maleimide Activated Horseradish Peroxidase Kit (Pierce, Cat.31494). Briefly, one mg of IgG was incubated with 80 ug of N-succinimidyl-S-acetylthioacetate for 30 min. After deacetylation with 5 mg of hydroxylamine HCl, modified IgG was ~~separeted by~~ separated using a polyacrylamide desalting column. ~~Protein pool~~ The protein pool was mixed with one mg of ~~maleimide activated~~ maleimide-activated horseradish peroxidase ~~was~~ and incubated at room temperature for 1 hr.

ii) Quantitation of OCIF by sandwich EIA

Microtiter plates (Nunc MaxiSorp Immunoplate) were coated with rabbit anti-OCIF IgG by incubating 0.2 ug in 100 ul of 50 mM sodium bicarbonate buffer pH 9.6 at ~~4C~~ 4°C overnight. After blocking the plates by incubating for 1 hour at 37℃ with 300 ul of 25% BlockAce/PBS (Snow Brand Milk Products), 100ul ~~of~~ samples were incubated for 2 hours at room temperature. After washing the plates three times with PBST (PBS containing 0.05% Tween20), 100 ul of 1:10000 diluted horseradish ~~peroxidase labeled~~ peroxidase-labeled anti-OCIF IgG was added and incubated for 2 hours at room ~~temperture~~ temperature. The amount of OCIF was determined by incubation with 100 ul of a substrate solution (TMB, ScyTek Lab., Cat.TM4999) and measurement of the absorbance at 450 nm using an ImmunoReader (Nunc NJ2000). Purified recombinant OCIF was used as a standard protein and a typical ~~standared~~ standard curve ~~was~~ is shown in Fig. 13.

EXAMPLE 25

Anti-OCIF monoclonal antibody

a

i) Preparation of hybridoma producing anti-OCIF monoclonal antibody.

the

OCIF was purified to homogeneity from culture medium of human fibroblasts,

cells Example

IMR-90 by the purification method described in Eample 11. Purified OCIF was dissolved in PBS at a concentration of 10 $\mu$g/100 $\mu$l. BALB/c mice were

administering

immunized by administrating this solution intraperitoneally three times every

was

two weeks. In the first and the second immunizations, the emulsion composed of an equal volume of OCIF and Freund's complete adjuvant was administered.

immunization removed and

Three days after the final administration, the spleen was taken out, lymphocytes were isolated and fused with mouse myeloma p3x63-Ag8.653 cells

conventional methods

according to the conventinal method using polyethyleneglycol. Then the fused

hybridomas

cells were cultured in HAT medium to select hybridoma. Subsequently, to check

The presence of

whether the selected hybridomas produce anti-OCIF antibody, anti-OCIF antibody

the each hybridoma Briefly,

in each culture medium of hybridomas was determined by solid phase ELISA which.

of a immunoplate was coated

was prepared by coating each well in 96-well immunoplates (Nunc) with 100 $\mu$l

blocked

of purified OCIF (10 $\mu$g/ml in 0.1 M $NaHCO_3$) and by blocking each well with 50% BlockAce (Snow Brand Milk Products Co. Ltd.). The hybridoma clones secreting

by limit dilution cloning 3-5 times

anti-OCIF antibody were established by cloning 3 - 5 times by limit dilution

screening.

and by screening using the above solid phase ELISA. Among thus obtained

Several hybridoma clones producing high levels of anti-OCIF

hybridoma clones, several hybridoma clones with high production of anti-OCIF antibody were selected.

ii) Production of anti-OCIF monoclonal antibodies.

Each hybridoma clone secreting anti-OCIF antibody, which was obtained in

EXAMPLE 25-i)[,] was transplanted intraperitoneally into [to] mice given Pristane (Aldrich), at a cell density of 1 x $10^6$ cells/mouse. The accumulated ascites was collected 10 - 14 days after transplantation, thereby obtaining [the transplantation and the ascites containing] anti-OCIF specific monoclonal antibody of the present invention [was obtained]. Purified antibodies were obtained by Affigel protein A Sepharose

chromatography (BioRad) according to the manufacturer's [maufacturer's] manual. Briefly, [That is] the ascites fluid was diluted with an equal volume of a binding buffer (BioRad) and applied to a protein A column. The column was washed with ~~a~~ sufficient volume of [the] binding buffer and eluted with an elution buffer (BioRad). After neutralizing, the eluate obtained [obtained eluate] was dialyzed in water and subsequently lyophilized. The purity of the antibody thereby obtained [obtained antibody] was analyzed by SDS/PAGE and a homogenous band with a molecular weight of about 150,000 was detected.

iii) Selection of monoclonal antibodies [antibody] having high affinity for [to] OCIF

Each antibody obtained in EXAMPLE 25-ii) was dissolved in PBS and the protein concentration [concentration of protein in the solution] was determined by the method of Lowry. Each antibody solution was diluted to [with] the same concentration [was prepared] and then serially diluted with PBS. Monoclonal antibodies, which can recognize OCIF even at highly dilute concentrations [diluted solution], were selected by solid phase ELISA described in EXAMPLE 25-ii). Thus, three monoclonal antibodies A1G5, E3H8 and D2F4 were [can be] selected.

iv) Determination of class and subclass of antibodies

The class and subclass of the antibodies of the present invention obtained in EXAMPLE 25-iii) were analyzed using an immunoglobulin class and subclass analysis kit (Amersham). The procedure was carried out according to the ~~protocol disclosed in the~~ Kit directions. The results ~~were~~ are shown in Table 15. The antibodies of the present invention, E3H8, A1G5 and D2F4 belong to the $IgG_1$, $IgG_{2a}$ and $IgG_{2b}$ subclasses, respectively.

Table 15

Analysis of class and subclass of the antibodies ~~in~~ of the present invention.

| Antibody | $IgG_1$ | $IgG_{2a}$ | $IgG_{2b}$ | $IgG_3$ | IgA | IgM | $\kappa$ |
|---|---|---|---|---|---|---|---|
| A1G5 | − | + | − | − | − | − | + |
| E3H8 | + | − | − | − | − | − | + |
| D2F4 | − | − | + | − | − | − | + |

v) ~~Determination~~ Quantitation of OCIF by ELISA

Three kinds of monoclonal antibodies, A1G5, E3H8 and D2F4~~, which were~~ obtained in EXAMPLE ~~25-iv)~~ 25-iv, were used as solid phase antibodies and enzyme-labeled antibodies, respectively. Sandwich ELISA was constructed by ~~each combination~~ different combinations of solid phase antibody and labeled antibody. The labeled antibody was prepared using an Immuno Pure ~~Maleimide Activated~~ Maleimide-Activated Horseradish Peroxidase Kit (Pierce, Cat. No. 31494). Each monoclonal antibody was

dissolved in 0.1 M $NaHCO_3$ at a concentration of 10 μg/ml, and 100 μl of the solution was added to each well ~~in~~ of a 96-well immunoplate~~s~~ (Nunc, MaxiSorp Cat. No. 442404) followed by allowing them to stand at room temperature overnight. Subsequently, each well ~~in~~ of the plates was blocked with 50% Blockace (Snow Brand Milk Products, Co., Ltd.) at room temperature for 50 minutes, and ~~then~~ ~~was~~ washed three times with PBS containing 0.1% Tween 20 (washing buffer).

A series of concentrations of OCIF was prepared by diluting OCIF with 1st reaction buffer (0.2 M Tris-HCl buffer ~~bufer~~, pH 7.4, containing 40% Blockace and 0.1% Tween 20). Each well ~~in~~ of a 96-well ~~immunoplates~~ immunoplate was filled with 100 μl of the prepared OCIF solution with each concentration, allowed to stand at 37 ℃ for 3 hours, and subsequently washed three times with ~~the~~ washing buffer. ~~For dilution of POD-labeled antibody, 2nd reaction buffer (0.1 M Tris-HCl buffer, pH 7.4, containing 25% Blockace and 0.1% Tween 20) was used.~~ The POD-labeled antibody was diluted 400-fold with 2nd reaction buffer (0.1 M Tris-HCl buffer, pH 7.4, containing Blockace a[nd] 0.1% Tween 2[0]), and 100 μl of the diluted solution was added to each well ~~in~~ of the immunoplates. Each ~~imunoplate~~ immunoplate was allowed to stand at 37 ℃~~C~~ for 2 hours, and subsequently washed three times with ~~the~~ washing buffer. After washing, 100 μl of a substrate solution (0.1 M citrate-phosphate buffer, pH 4.5, containing 0.4 mg/ml of o-phenylenediamine HCl and 0.006% $H_2O_2$) was added to each well ~~in~~ of the immunoplates and the immunoplates ~~were~~ incubated at 37℃ for 15 min. The enzyme reaction was terminated by adding 50 μl of 6 N $H_2SO_4$ to each well. The optical density of each well was determined at 492 nm using an immunoreader (ImmunoReader NJ 2000, Nunc).

Using three ~~kinds of~~ different monoclonal ~~antibody in~~ antibodies of the present invention, each

combination of solid phase and POD-labeled antibodies leads to a an accurate determination of concentration OCIF. Each monoclonal antibody in of the present invention was confirmed to recognize a different epitope of OCIF. A typical standard curve of OCIF using a combination of solid phase antibody, A1G5, and POD-labeled antibody, E3H8 was is shown in Fig. 14.

vi) Determination of OCIF in human serum

The concentration Concentration of OCIF in five samples of normal human serum was determined using an EIA system described in EXAMPLE 25-v). The immunoplates were coated with A1G5 as described in EXAMPLE 25-v), and 50 $\mu$l of the 1st. reaction buffer was added to each well in of the immunoplates. Subsequently, 50 $\mu$l of each human serum was added to each well in of the immunoplates immunoplates. The immnuoplates were incubated at 37℃ for 3 hours and then washed three times with the washing buffer. After washing, each well in of the immunoplates was filled with 100 $\mu$l of POD-E3H8 antibody diluted 400-fold with the 2nd. reaction buffer and incubated at 37℃ for 2 hours. After washing the immunoplates three times with the washing buffer, 100 $\mu$l of the substrate solution described in EXAMPLE 25-v) was added to each well and incubated at 37℃ for 15 min. The enzyme reaction was terminated by adding 50 $\mu$l of 6 N $H_2SO_4$ to each well in of the immunoplates. The optical density of each well was determined at 492 nm using an immunoreader (ImmunoReader NJ 2000, Nunc).

1st. reaction buffer containing the known amount of OCIF was treated in the same way and a standard curve of OCIF as shown in fig. 2 was obtained. Using the standard curve of OCIF, the amount of OCIF in human serum sample was

determined. The results were shown in Table 14.

Table 14

The amount of OCIF in normal human serum

| Serum Sample | OCIF Concentration (ng/ml) |
|---|---|
| 1 | 5. 0 |
| 2 | 2. 0 |
| 3 | 1. 0 |
| 4 | 3. 0 |
| 5 | 1. 5 |

## EXAMPLE 26

Therapeutic effect on osteoporosis

(1) Method

Six week old male — the

Male Fischer rats, 6 weeks-old, were subjected to denervation of left forelimb. These rats were assigned to four groups(10 rats/group) and treated as follows ; group A, sham operated rats without administration ; group B,

the vehicle administered intravenously

denervated rats with intravenous administration of vehicle ; group C,

with OCIF administered

denervated rats administered OCIF intravenously at a dose of 5 μg/kg twice

with OCIF administered

a day ; group D, denervated rats administered OCIF intravenously at a dose of 50 μg/kg twice a day. After denervation, OCIF was administered daily for 14 days. After 2 weeks treatment, the animals were sacrificed and their forelimbs were dissected. Thereafter bones were tested for mechanical

strength.

(2) Results

Adecrease in Control animals

Decrease of bone strength was observed in the animals of control groups as compared to those animals of the normal groups while bone strength was

Group animals that

increase in the groups of animal received 50 mg of OCIF per kg body weight.

## Industrial availability

The present invention provides both a novel protein which inhibits the

an for producing

formation of osteoclasts and a efficient procedure to produce the protein.

inhibits the

The protein of the present invention has an activity to inhibit formation of osteoclasts. The protein will be useful for the treatment of many diseases

accompanied by to prepare antibodies

accompanying bone loss, such as osteoporosis, and as an antigen to be used for useful the immunological diagnosis of such diseases.

Referring to the deposited the microorgainsm microorganism

Name and Address of the Depositary Authority

Name: National Institute of Bioscience and Human-Technology
Agency of Industrial Science and Technology
Ministry of International Trade and Industry

Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken
305, JAPAN

Deposited date: June 21, 1995

(It was transferred from Bikkoken No. P-14998, which was deposited on June 21, 1995. Transferred date: October 25, 1995)

Acession Number: FERM BP-5267